Využití ultraúčinné kapalinové chromatografie ve spojení s hmotností spektrometrií v metabolomické analýze plasmy

Diplomová práce

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Hradec Králové 2012 *Karolína Machová*

Metabolomic analysis of plasma samples using ultra performance liquid chromatography and high resolution mass spectrometry

Diploma thesis

UNIVERSITY OF EASTERN FINLAND
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„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.“

V Hradci Králové 2012 Karolína Machová

Contents

Abbreviations

ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
C18	Oktadecyl carbon chain column
Cpd.	Compound
DC	Direct current voltage
EI	Electron ionization
EIC	Extracted Ion Chromatogram
ESI	Electrospray ionization
F5	Pentafluorophenylpropyl column
Fig.	Figure
FT-ICR	Fourier transform-ion cyclotron resonance
GC	Gas chromatography
HILIC	Hydrophilic-Interaction Liquid Chromatography
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption ionization
MFE	Molecular feature extraction
min	Minute
MS	Mass Spectrometry
NMR	Nuclear magnetic resonance
ppm	Parts Per Million
Q-TOF	Quadrupole–time of flight
RF	Radiofrequency
RPC	Reversed phased chromatography
RT	Retention time
SIM	Selected ion monitoring
Tab.	Table
TOF	Time-of-flight
UPLC	Ultra-performance liquid chromatography

Abstrakt

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Metabolomika je nová věda popisující komplexní metody pro určování a měření metabolitů v biologických vzorcích především tělních tekutinách jako je plasma, moč, sérum, sliny, cerebrospinální tekutina, sperma. Popisuje děje probíhající v buňkách a nachází široké uplatnění v diagnostice onemocnění, určování biomarkerů, patofyziologii nemocí a klinické praxi.

Hladiny metabolitů jsou v nízkých koncentračních rozmezích, a proto je kladen důraz na vysokou citlivost metody. Důležitá je reprodukovatelnost analytické metody a také příprava vzorku. Metabolomická analýza využívá pro stanovení metabolitů především vysokoúčinnou kapalinovou chromatografii v kombinaci s hmotnostním detektorem. Další běžné analytické metody jsou nukleární magnetické rezonance a plynová chromatografie.

Následující projekt je zaměřen na standardizaci metody identifikace nalezených metabolitů pomocí ultra-účinné kapalinové chromatografie v kombinaci s hmotnostním spektrometrem s využitím počítačového vyhodnocení pomocí databáze známých látek.

Abstract

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Diploma Thesis Title: Metabolomic analysis of plasma samples using ultra performance liquid chromatography and high resolution mass spectrometry

Metabolomics is a novel type of science which represents a complex methods of metabolite determination in biofluid e.g. plasma, urine, saliva, cerebrospinal fluid and sperm. The method describes cell mechanisms and finds wide assertion in disease diagnostics, biomarkers description, disease, pathophysiology and in clinical practice.

It is vital to have a sensitive method because levels of metabolites are quite low. Metabolomic analysis uses mainly high-performance liquid chromatography combined with mass spectrometry for detection. However, other methods such as nuclear magnetic resonance spectroscopy and gas chromatography can also be used.

Presented work is mainly focused on method standardization for identifying metabolites in clinical practice with the use of ultra-performance liquid chromatography and mass spectrometry. The primary outcome was to establish searching parameters for the operating software to automatically identify xenobiotics in provided sample and compare them with existing database. Time efficient, reproducible and sensitive method was to be suggested for clinical practice.

Aim of the thesis and assignment of the work

The aim of presented thesis was to develop and standardize method for identifying samples and comparing them with their signatures in database of several thousand well-known substances. Proposed method would be an efficient way to analyze samples of biofluids (plasma, urine, etc.) and should be adapted in clinical practice. The analysis used UPLC with appropriate column displaying suitable attributes. Also finding the best settings for the analysis, interpreting and comparing the results from the MS detector correctly and comparing them with chosen database is an essential part of developing a novel method.

PART 1 - Theory

1 Metabolomics

Metabolomics deals with exploration of the metabolom. Metabolom is set of all intra- and extracellular low molecular weight compounds (e.g. lipids, amino acids, peptides, nucleic acids, organic acids, vitamins) in live systems, which participate in metabolic reactions. These compounds are necessary for physiological growth and functionality of cells. (1)

It is a relatively new science, which describes a complex method for determination and measuring biological sample metabolites, e.g. biofluids (blood, serum, urine, saliva, cerebrospinal fluid, synovial fluid, semen). Practical assertion could be physiological status investigation, disease diagnostic tool, biomarkers determination or false metabolic pathways discovery due to disease or treatment. (2) Metabolomics has unsubstitutable importance in pharmaceutical innovative development. Also it is a prospective method for mapping of divergence early biochemical changes in disease and acquiring valuable knowledge on the diseases mechanisms.

Metabolites can be analyzed by standard tools of chemical analysis. Common analytical techniques used in metabolomics are nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), gas chromatography (GC) and liquid chromatography (LC) hyphenated with mass spectrometry. Each technique has poses advantages and disadvantages. Choice of the most appropriate method depends on the character of the analyzed sample. NMR and MS with direct infusion of the sample into the ion source are used mainly when the aim is to analyze as many metabolites without identification and quantification. For better characterization of metabolome including identification and quantification separation analytic methods such as GC and HPLC hyphenated with MS detector are used. (1) (2) (3) (4)

1.1 Metabonomics

Metabonomics is a part of metabolomics, widely used in the drug discovery and development, which studies the metabolic response to drug administration and presence of toxic substances in the tissues and body fluids. HPLC-MS helps to develop more detailed understanding of metabonomics. The focus is aimed at the characterization of physiologically important metabolites, mainly their structure basic biochemistry and biomarker discovery. (1) (3)

2 LC/MS - Liquid Chromatography coupled with Mass Spectrometry

A combination of LC and MS allows emergence hyphenated technique, which offers an advantageous exploiting of both techniques - LC as a universal separation technique and MS as a sensitive detection and identification technique.

In the past years LC-MS found a place in many application areas e.g. in environmental, food safety, pharmaceutical drug discovery and development, clinical analysis etc. and still is developing. (5)

In the 30 years past there were three objectives which were achieved:

- the ability to couple conventional LC columns to a mass spectrometer
- the ability to achieve analyte enrichment in order to enable electron ionization (EI)
- the ability to ionize analytes directly from the liquid phase

Now LC-MS is widely used and applied to finds olutions to many analytical assignments, ionic and highly polar analytes and even biomacromolecules, are now utilizable to MS analysis. (5)

2.1 High-Performance Liquid Chromatography

Liquid chromatography (LC) is an essential technique. In contrast to gas chromatography, which is used for separation of volatile and thermally stabile molecules, liquid chromatography can separate a lot of organic compounds, from small-molecule drug metabolites to peptides and proteins. (6)

2.2 Mass Spectrometry

Mass spectrometry (MS) is a standard detector system for analysis of pharmaceutical compounds in biological systems like plasma or urine. MS detectors are also used to describe structural information or confirmation of unknown substances, although it does not have the mass-resolution capability of traditional stand-alone mass spectrometers. (7). They are very useful to obtain mass spectral data which are weight, structure, identity, quantity and purity of a sample. Mass spectral data are important for qualitative and quantitative analyses. (6)

2.3 **Interfaces**

The development of the MS detector interface is an important factor in combination of mass spectrometry as HPLC detection. MS detectors detect ions only in gaseous phase, so it is necessary to evaporate the mobile phase and then ions have to be generated. This is the function of the MS detector interface. The mobile phase passes from liquid to gas phase. Its volume grows rapidly; so there is a need to lower the pressure to 10^{-5} - 10^{-6} torr. The pressure is reduced by pumping the most of the vaporized sample and mobile phase to waste and only a lesser sample fraction moves into the MS. Several varieties of interfaces were developed during past years. Now the problem is solved selecting two most used interfaces, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), both based on atmospheric-pressure ionization principle. (5) (7)

3 High-Performance Liquid Chromatography

The basic principle of chromatography is a separation of compounds between two immiscible phases. Mobile phase is delivered into the system by the high-pressure pump and transported to the column where it flows through the stationary phase and the separation takes place. (5) (7)

Separation is influenced by several parameters. Separation efficiency is a function of stationary phase particle size. In relationship with particle size is a separation pressure. The smaller the particles are, the higher pressure for separation is necessary. Degasser is liable for clearing the mobile phase of improper gasses. Purity of solvent is required in light of results quality - contaminated dissolvent makes false background in chromatogram. (5) (7)

There are two composition of mobile phase as analysis runs - isocratic elution when the quality of solvent is the same throughout the separation, and gradient elution when the composition of the solvent change during the separation (5) (7)

The particles of stationary phase are mostly bind on porous silica organized as cylinders of specific diameter inside of the column. According to bound stationary phase attached to the silica particles several types of separation modes are recognized. (5) (7)

- Reverse-phase chromatography. Column is nonpolar. Mobile phase is polar mixture of water and organic solvents.
- Normal-phase chromatography. Column is polar. Mobile phase are less polar organic solvents.
- Ion-exchange chromatography. Column contains charged groups that can bind sample ions of opposite charges. Mobile phase is aqueous solution of salt plus buffer.
- Ion-pair chromatography. Column is nonpolar. Mobile phase contains ion pair reagent.

3.1 Ultra-Performance Liquid Chromatography

Ultra Performance Liquid Chromatography (UPLC) is a new separation technique in chromatographic methods. Separation process uses sorbents prepared by patented "bridge hybrid particle" method. They excel their mechanical consistence and extraordinary separation efficiency. The whole process proceeds using very high pressure (100 Mpa) (8)

UPLC brings several benefits in contrast to classic HPLC technique:

- Time of analysis is shorten, because UPLC has higher productivity
- Lower financial cost
- Increased efficiency of separation
- Better sensibility
- More qualitative results (8)

3.2 Columns

Column is a metal cylinder which contains silica particles with stationary phase inside. The column is the heart of the LC system. Stationary phase determines retention and selectivity. Particle quality and size determine efficiency.

3.2.1 Hydrophilic-Interaction liquid Chromatography column

Hydrophilic interaction chromatography (HILIC) is a method performed on LC, silica column is not modified and it uses an aqueous-organic mobile phase. It is an opposite to reverse phase because highly polar analytes are held on column and are eluted as last. If ESI-MS is used, basic compounds are eluted by acidic mobile phase and detected in the positive-ion mode, acidic analytes used neutral pH and detected in the negative-ion mode. An advantage is used with analytes poorly retained in reversed-phase LC because they show good retention in HILIC. (5)

HILIC characteristic include several positive features:

- good peak shape for basic solutes
- enhanced mass-spectrometer sensitivity
- possibility of direct injection of samples that are dissolved in a primarily organic solvent (which would be unsuitable for RPC)
- higher flow rates (or lower column pressures) possible, because of the lower viscosity of the mobile phase

Almost all HILIC columns use silica particles as standard. Subsequently they vary of different bonded-silica packings form HILIC. They can be categorized as follows: bare silica, polar neutral (e.g., cyanopropyl), diol-bonded, amide-bonded, polypeptide-bonded, positively charged amine-bonded (anion-exchange), negatively charged (cation-exchange), and zwitterionic phases (7)

3.2.2 **Fluorine column**

The pentafluorophenylpropyl stationary phase of Ascentis Express F5 provides a stable reversed packing with electron-deficient phenyl rings due to the presence of electronegative fluorines. In addition to forming pi-pi and mildly steric interactions, F5 phases also retain compounds by polar interactions. Ascentis Express F5 can be used for basic, acidic, or neutral compounds with alternate selectivity from C18. (9)

The stationary phase is fluorinated silica-based. The basic structural unit is a pentafluorophenyl ring which is attached to the silica over a propyl chain. The PFPP has increased retention. The stationary phase provides separations that are different from C18. F5 sufficiently retains compound that C18 column elute too closely. It is caused by mixed reversed and normal-phase retention mechanism selectivity. It allows co-temporary elution of polar and non-polar compounds. (10)

4 Mass spectrometry

4.1 Instrumentation

Mass spectrometers (MS) work by ionizing molecules and then sorting and identifying them according to their mass-to-charge (*m/z*) ratios. The resulting mass spectrum is a report of the abundance of the generated ions from surveyed sample as a function of the *m/z*. (5)

A basic requirement for atoms or molecules is that they have to be ionized and analyzed as gas phase ions. (11)

The mass spectrometer is a highly complex and computerized device. It consists of five parts:

- sample introduction
- ionization source
- mass analysis
- ion detection
- data handling. (5)

Two crucial components in this process are the ion source, which generates the ions, and the mass analyzer, which sorts the ions. (6) Indisseverable part of MS is a computer software that operates the whole detection process and is liable to evaluate data.

4.2 Sample introduction

Mechanism of introducing the sample into the device is significant. The first component is the sample inlet that is connected e.g. to HPLC or GC. The sample is introduced to the ion source and before entering to mass analyzer must be ionized. There are two types of sample introduction to the ion source:

- direct injection
- insertion using an insert when the samples is applied on to a surface. So that is physically placed in the source. This is used with MALDI systems (matrix-assisted laser desorption ionization) (12)

4.3 Ionization methods

Ionization methods transfer ions from compounds. Accessible methods for ionization are protonation, deprotonation, electron ejection, electron capture, cationization, anionization and transfer of a charged molecule to gas phase (13).

4.4 Ion sources

The function of the ion source is to introduce molecules into the mass spectrometer and transfer them to ionized form. The ion source is working at low pressure unlike mass spectrometer which is under vacuum. Vacuum helps to prevent the collision of ions with residual gas molecules when they are transferred to the detector. (14)

There are many types of common ionization techniques, but they can be divided into two main groups as hard or soft. It depends on the latitude of fragmentation (amount of energy inserted to the compound) during the ionization process. Electron ionization (EI) is an example of a hard ionization method, while the currently extensively applied electrospray ionization and matrix-assisted laser desorption ionization (MALDI) are soft ionization techniques. (5)

Most common source is atmospheric pressure ionization (API) technique. Implementing this method to analysis extended amount of compound that could be analyzed by LC/MS. Earlier were molecules ionized in the mass spectrometer under vacuum (e.g. by electron ionization) and limited number of compounds fit these conditions. By API molecules are ionized at atmospheric pressure and ions are after mechanically and electrostatically separated from neutral molecules.

API group consist of:

- Electrospray ionization (ESI)
- Atmospheric pressure chemical ionization(APCI)
- Atmospheric pressure photoionization (APPI) (6)

API techniques generate several particles:

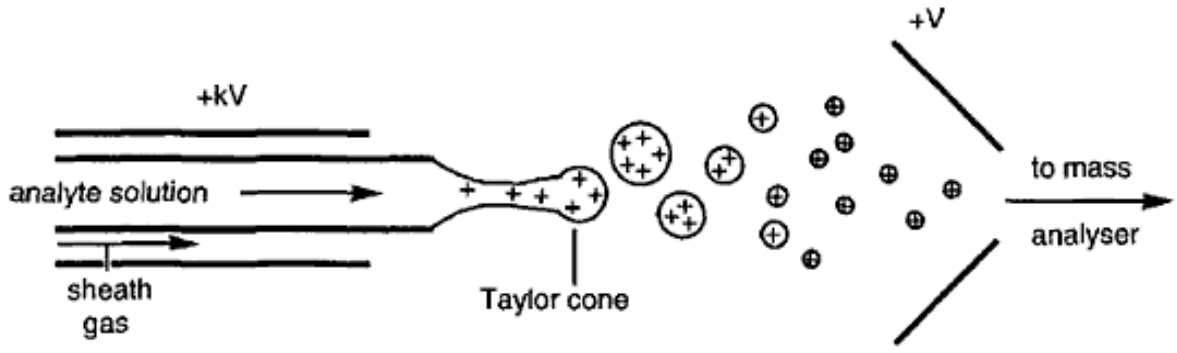
- molecular ion M^+ and M^-
- protonated molecules $[M + H]^+$
- simple adduct ions $[M^+ + Na]^+$

4.4.1 Electrospray ionization

Electrospray ionization (ESI) is example of atmospheric pressure ionizatio n source and is considered as a so ft ionization technique. Basic principle is that eluent is sprayed by a needle at an atmospheric pressure in a strong electrostatic field an d heated. Small ch arged droplets l eave t he apex of t he ne edle a nd f low t o t he c ounter electrode. D uring t he pa ss droplets r educe t heir mass and s ize be cause t hey a re dried. C oncentration of c harge (Columbic r epulsive f orces) overcomes liquid s urface t ension a nd di sintegrates droplets to smaller ones. Ions are attracted and pass through a capillary sampling orifice into MS where are detected. (6) (11) (12) (14) (15)

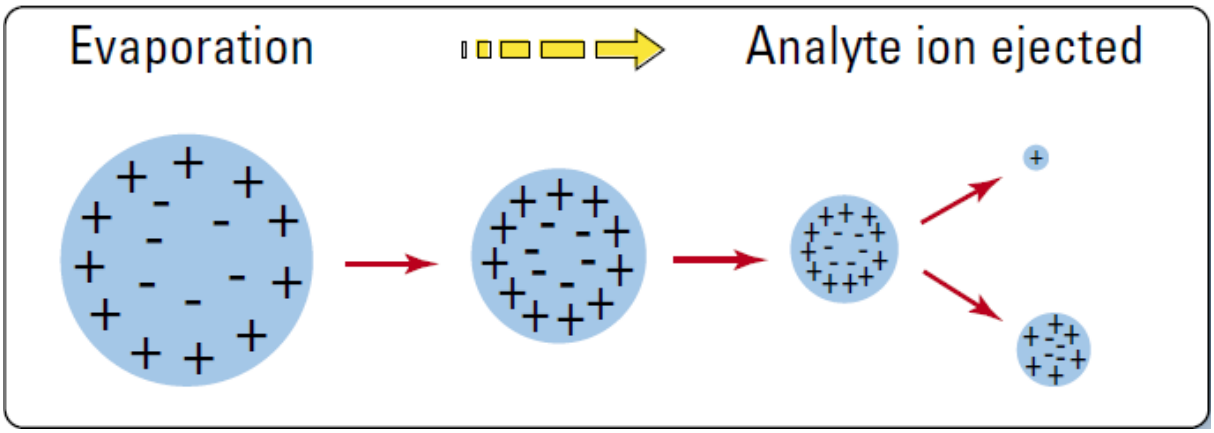
Electrospray i s us ed with a dvance i n l arger m olecules e .g. p roteins, pe ptides, oligonucleotides e ven t hough m ass r ange of LC-MS i s around 300 0 m /z a nd t he l arge molecules have value m/z around 150000 because molecules can obtain more than one charge (multiple-charging). (6) (11) (12) (14) (15)

Fig. 1: Schema of ESI principle



Downard, Kevin. Mass Spectrometry: A Foundation Course. Cambridge : The Royal Society of Chemistry, 2004. ISBN 0-85404-609-7

Fig. 2: Schema of ions formation



Basic of LC/MS. U.S.A. : Agilent technologies, 2001. 5988-2045EN

The sampling of ions requires severe pressure reduction. A gas flow introduced into a vacuum system expands and cools down. To obtain good spectra results there is an interface to prevent cluster formation, e.g. the space between the orifice and the curtain plate is flushed with heated pure nitrogen. Ions flow through the curtain gas into the mass analyzer attracted by an electric field. Neutral solvent molecules are not attracted and cannot penetrate into the high vacuum region, which prevents the formation of cluster ions. (11)

4.5 **Mass Analyzers**

Ions are speed in mass analyzer and separated according to their m/z. In principle mass analyzers can be divided according to the presence of magnetic and/or electric fields. An electrical current is produced as an ascent and get intensity by amplifier. (14)

Four types of MS are used most often:

- Quadrupole
- Time-of-flight
- Ion trap
- Fourier transform-ion cyclotron resonance (FT-ICR)

4.5.1 **Quadrupole analyzer**

Today the quadrupole mass analyzer the most common used. The advantages are relative high pressures tolerance, the capability of analyzing is up to 4000 m/z and they is simple and less expensive than other mass analyzers. (16)

A quadrupole mass analyzer consists of four parallel rods arranged in a square which use hyperbolic radiofrequency field arisen by brought voltage. The field is responsible for passing and filtering ions during the time. The analyzer is operating in two mods - Scanning and Selected ion monitoring mode (SIM). Mods are function of type of electic field. SIM mode is used for quatification of known compounds and targeted ions. On the other hand Scan mode is suitable for monitoring all analyte masses unknown in advance. (6)

The rods are concurrently placed, opposite rods have the same direct current (DC) potential with an oscillating radiofrequency alternating current (RF). Only ions of selected m/z or its interval can pass through to the detector. The others are moved from electric field to the rods. By continuous changing RF and DC all ions are analyzed in selected m/z (5) (11).

4.5.2 **Time-of-flight analyzer**

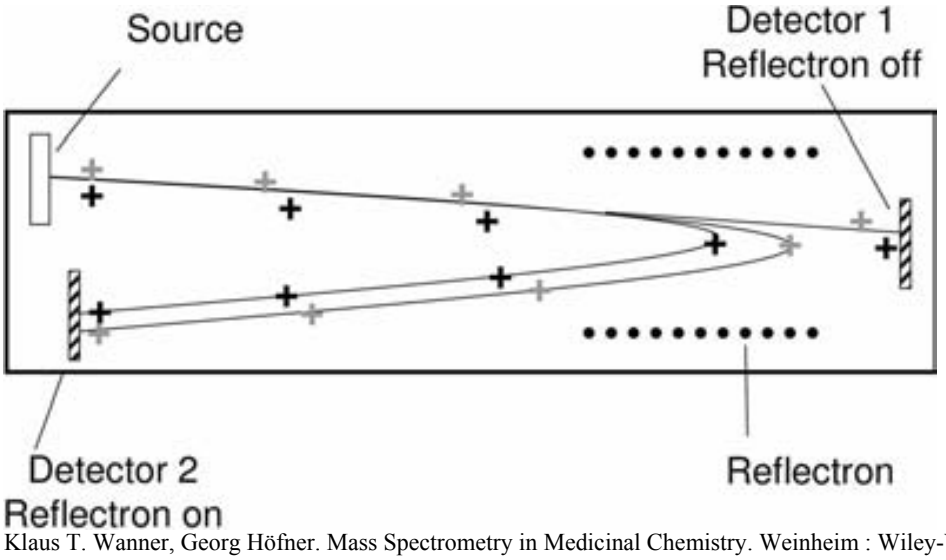
Time-of-flight (TOF) mass analyzer works on the principle of measuring the time which ions needs in tube about 1m in length, field free, to arrive to the detector. Time depend on m/z value. Ions with low mass reach the detector faster than high mass ions. Ions are accelerated at the same time by uniform electromagnetic field in the direction of the detector. The time of flight is function of the energy used for acceleration, the distance it has to travel, and its m/z value. (5) (6) (11) (16) (17)

Characteristic quality of TOF mass analyzer is the theoretical unlimited mass range, but practically the system is limited to m/z 10,000 or 20,000. Mass resolution is poor because of the kinetic energy of the ions leaving the ion source and it is also limited by the length of the flight tube. The kinetic energy and the length of the tube are factors affecting distribution in the flight time of ions with the same m/z. The simplest way to improve the mass resolutions is to increase the length of the tube and reduce the kinetic energy spread of the ions leaving the source. (5) (6) (11) (16) (17)

Double the length of the tube can be obtained using an ion mirror reflectron (electrostatic mirror), this upgrade will result in improved separation between ions with different m/z. The reflectron consists of several lens plates of different voltage and are decelerated for return to the detector. (5) (6) (11) (16) (17)

The TOF reflectron used together with ESI, which provides continual ions inflow, is preferred due to higher resolution, fast scanning capabilities, good mass range, and an accuracy in the order of 5 ppm. (16)

Fig. 3: Schema of Time-of-fligh reflectron



Klaus T. Wanner, Georg Höfner. Mass Spectrometry in Medicinal Chemistry. Weinheim : Wiley-VCH, 2007. ISBN 978-3-527-31456-0

4.5.3 **Q-TOF analyzer**

Q-TOF mass analyzers are used as a hybrid instrument quadrupole–time of flight (Q-TOF) in tandem mass spectrometry because of their acquisition rate and high resolution capabilities. It is a combination of Triple-Quadrupole mass spectrometer and Time-of-flight mass spectrometer, the third Quadrupole in Tandem type is replaced by TOF. The analyzer can operate in single MS mode when quadrupoles serve as ion guide and analysis is performed in the TOF. The second is tandem MS mode. (5) (11)

5 MassHunter Qualitative Analysis software

Molecular feature extraction (MFE) algorithm is a compound finding technique that locates individual sample components (molecular features), even when chromatograms are complex and compounds are not well resolved. MFE locates ions that rise and fall together in abundance but the analysis is not exclusively based on chromatographic peak information. The algorithm uses accuracy of the mass measurements to group related ions (related by charge) state envelope, isotopic distribution, and presence of adducts and dimmers. It assigns multiple species (ions) that are related to the same neutral molecule to a single compound that is referred to as a feature. Using this approach, the MFE algorithm can locate multiple compounds within a single chromatographic peak. (18)

The program uses SCORE value for identification accuracy. Program makes list of candidates with the same molecular weight and assigns it to the molecular formulas and adds probability in percent that characterize validity of the result.

PART 2 - Experimental

6 Instrumentation

6.1 Equipment

All samples were analyzed on 1290 Infinity UPLC system with wellplate sampler, binary pump, thermostatted column, diode array detector compartment, coupled with a 6540 UHD Accurate–Mass Q-TOF mass spectrometer system with dual ESI source, operated in the positive-ion mode. ESI capillary voltage was set at 4000 V and fragmentor at 100 V for plasma sample measured on F5 column and 140 V for standard mixture sample and HILIC column. The liquid nebulizer was set to 45 psig and the nitrogen drying gas was set to a flow rate of 10 l/min. The drying gas temperature was maintained at 325 °C. The acquisition rate was 1,5 spectra/sec (2,4 spectra/sec for HILIC column) and a stored mass range of m/z 50-1600.

MassHunter Workstation Data acquisition software was used to operate the instrumentation. Data were processed using MassHunter Qualitative Analysis software (Agilent Technologies). Compounds were extracted from data using the Molecular Feature Extraction algorithm in Mass Hunter Qualitative analysis software. Compound identification was performed using Molecular Formula Generation algorithm and Metlin Personal Metabolite Database prepared from Metlin Database used MassHunter PCDL Manager.

- Mass spectrometer:
  Agilent Technologies 6540 UHD Accurate–Mass Q-TOF LC/MS

- UPLC:
  Agilent Technologies 1290 Infinity

- Program:
  Qualitative Analysis B.04.00 MassHunter Acquisition Data
  MassHunter PCDL manager

- Other:
  shaking - Vortex
  Centrifuge - Eppendorf 5415 R
  Evaporation - vacuum, Jouan RC 10.10., 55 ˚C

6.2 **Chemicals and Materials**

- Plasma sample:
  human plasma B^+

- Chemicals:
  Methanol (J.T. Baker., 8402, 2,5)
  Acetonitrile (VWR PROLABO, 83640.320)
  Formic acid (Fluka Analytical, 98%, 1l, 06450)

- Distilled water:
  MILLIPORE, Milli-Q®, Gradient , A10, PMC-LA-0034, Quantum®EX, Ultrapure Organex Cartridge, LOT - FOPA84536, 18,2 (18,3) MΩ.cm,25 ˚C, TOC: 3 ppb

- Filters:
  syringe filter Agilent 5061-3366 (syringe without needle, 1ml)
  Microcon centrifugal filter devices ultracel YM-50 Rose (regenerated cellulose 50000 MWCO, membrane NMWL 50000, maximum G-force 14000)
  Millex® -HV (syringe Driver Filter unit non-sterile, 0,45µm, 13mm, lat. NO SLHVO13NK, Lot R9EN97841)

- Syringe:
  1ml without needle (Termo company)

- Vials:
  plastic – 1,5 ml
  glass – HPLC, Part number 5182-0715
  vial insert – P.N. 5183-2085, 250 µl

- Pipets:
  VoluMate Liquisystems, Mettler Toledo (0,5-10µl, 10-100µl, 20-200µl,100-1000µl)
  Finnpipette, Labsystems (1-5 ml, J38684)

6.3 **F5 column**

Chromatographic separation was achieved on a 5 cm x 2,1 mm, 2,7-µm particle size F5 HPLC column (Ascentis Express. Col: 122514-05).

LC parameters: Solvent A was 0,2% acetic acid and solvent B methanole in 0,2% acetic acid. The flow rate was 0,4 ml/min, the solvent gradient program is shown in Table 1. Stop time was 19 min. The injection volume was 1 µl for plasma sample and 2µl for standard mixture sample. Column temperature was set at 40°C.

Tab. 1: F5 column solvent gradient settings

Time (min)	Solvent Ratio B
00,0	2
13,0	98
15,0	98
15,1	2
19,0	2

6.4 **Hydrophilic-Interaction liquid Chromatography column**

Chromatographic separation was achieved on a 2,1 x 100 mm, 1,7-µm particle size Hilic Acquity UPLC ® B EH Amide column (Par. No. 186004801, LOT No. 0116312451) LC parameters: Solvent A was 50% acetonitrile in 20mM ammonium formate and solvent B 90% acetonitrile in 20mM ammonium formate. The flow rate was 0,6 ml/min and the solvent gradient program is shown in Table 2. Stop time was 19 min. The injection volume was 1 µl and column temperature was set at 45°C.

Tab. 2: HILIC column solvent gradient settings

Time (min)	Solvent Ratio B
00,0	100
02,5	100
10,0	0
14,5	0
14,6	100
18,0	100

7 Screening experiments

The screening experiments included the LC/MS analyses and data processing of human plasma B^+ sample and standard mixture created from aminoacids and some few other substances present in human plasma.

7.1 Sample preparation

For adjust sample was used a technique of solvent precipitation for proteins separation. All samples were prepared three times.

7.1.1 F5 column

Samples were prepared in two different pH. First in pH=7 and second one in lower pH. First step was to dilute plasma. For normal pH was used 50 µl plasma with 50 µl of distilled water and for lower pH 50 µl plasma and 50 µl 0,2 % formic acid. Other steps were same for both ways.

400 µl of methanol was added; shaken for 10 seconds with Vortex and then left it 20 minutes in fridge. After centrifugation, it was used with settings 13000 rpm, 10 minutes. Then was filtrated 300 µl of supernatant with syringe filter to plastic vial and vacuum evaporated for 1 hour and 15 minutes. In the end it was dissolved with mixture of 50 µl of methanol and 50 µl of distilled water containing 0,1 % formic acid and transferred it into HPLC glass vials with vial insert.

Samples were not clear after filtration with the Agilent syringe filter, so the centrifugal filter was used. After 10 min at 13000 rpm small amount of filtrate was obtained, than continued next 35 minutes at 13200 rpm. In the end was clean filtrate transferred into glass vial with insert.

For control of filtration were prepared two blank samples, one contained 100 µl of distilled water, second one 50 µl of distilled water and 50 µl of 0,2% formic acid and they were filtrated with centrifugal filter 45 minutes at 13200 rpm.

7.1.2 **HILIC column**

Samples were adjusted by a dded 100 µl of plasma, diluted with 300 µl of cold acetonitrile, than shaken with Vortex, and kept in the fridge for 100 minutes. Then the sample was put into centrifuge for 15 minutes at 13200 rpm and after supernatant was filtrated with syringe filter into the HPLC vials with insert.

7.1.3 **Standard mixture**

Standard mixture of compounds was prepared according to article (19). Using micro scale was added in to glass vial exact amount of chemical and then added exactly 1 ml solvent per 1mg of chemical. Standard mix was prepared by mixing exactly 1,00 ml of each chemical dissolved into a 100 ml flask and added 2% acetonitrile in 0,1% formic acid to a final volume of 100 ml and mixed well.

Tab. 3: List of chemicals used for preparation of Standard mixture

	Name of Cpd	Cpd (mg)	Solvent (µl)	Solvent	Information
1	L-Tyrosine	0,99	990	10% ACN	Fluka Analytical 93830
2	Uracil	1,01	1010	10% ACN	Aldrich 22405-041
3	L-Pyroglutamic acid	1,3	1030	2% ACN	Fluka Biochemika 83160
4	L-Threonine	1,04	1040	2% ACN	Sigma-Aldrich 128K00192
5	2-L-Glutamine	1,4	1400	2% ACN	Fluka 96120
6	Nicotinic acid	1,33	1330	2% ACN	Sigma-Aldrich N4126-100G
7	L-Glutamine	1,25	1250	0,1% HCOOH	Merc 1114454 1250
8	L-Proline	1,08	1080	2% ACN	Fluka Biochemika 81710
9	p-Coumaric acid	1,51	1510	10% ACN	Fluka Biochemika 28200
10	2-Deoxy-D-ribosa	1,23	1230	2% ACN	Aldrich-chemistry 121649-5G
11	L-Lysine	1,41	1410	2% ACN	Sigma BCBC1966
12	Cystidine	1,55	1550	2% ACN	Sigma C4654-1G
13	Glycine	1,2	1200	2% ACN	Riedel-de Hane 33226
14	L-Cystine	1,34	1340	2% ACN	Sigma C8755100G
15	L-Aspartic acid	1,08	1080	2% ACN	Sigma A9256-100G
16	Nicotin amide	1,29	1290	0,1% HCOOH	Fluka 72340
17	L-Alanine	1,45	1450	2% ACN	Sigma A7627-100G
18	Malonic acid	1,38	1380	2% ACN	Aldrich S09041-073
19	Hippuric acid	1,1	1100	2% ACN	Aldrich 11020-114
20	Caffeine	1,41	1410	2% ACN	Aldrich 11020-114

21	L-Methionine	0,49	490	2% ACN	Sigma 63H0802
22	L-Isoleucine	1,09	1090	2% ACN	Sigma 46H14431
23	Tryptophan	1,29	1290	10% ACN	Sigma 126H03791
24	Adenosine-5-monophosphate	1	1000	10% ACN	Sigma 078K1492
25	L-Phenylalanine	1,34	1340	2% ACN	Sigma 32H0521
26	D-Glucose-6-phosphate	1,51	1510	2% ACN	Sigma 093K3789
27	L-Rhamnose	1,46	1460	2% ACN	Sigma 57H0567
28	L-Arabinosa	1,45	1450	2% ACN	Sigma 87H0721
29	D-Galactose	1,53	1530	2% ACN	Sigma 67H0561
30	γ-aminobutyric acid	1,37	1370	2% ACN	Sigma 026K07361
31	Guanidine	1,45	1450	2% ACN	Sigma 68H5437
32	Leucin enkephalin	1,08	1080	2% ACN	Sigma 38H5803
33	Standard mixture	1 ml 40 of each			

7.2 **Database**

Because Metlin database contains about 24000 compounds and this number is too high, the database of demanded substances was created. It was used as a source for searching of masses for mass filter.

Database was created from original Metlin database in program PCDL manager. Demanded compounds were highlighted, than there was chosen a possibility to create subset PCDL from highlighted compounds and saved it. Database included compounds used for standard mixture preparation, the other possible compounds in plasma from articles (18), and some polyamines. Database contains 97 compounds and values as Formula, Mass, RT, IUPAC name.

Tab. 4: List of compounds included into the database

Compound Name	Formula	Mass	RT (min)	METLIN
1,1-Cyclopropanedicarboxylic acid	C5H6O4	130.02661		3714
1,3-Propanediamine	C3H10N2	74.08440	0.272	3216
1-methylhistidine	C7H11N3O2	169.08513	0.328	3741
2-Aminoadipic acid	C6H11NO4	161.06881	0.363	324
2-Ketoglutaric Acid	C5H6O5	146.02152	0.423	119
2-Methylhippuric acid	C10H11NO3	193.07389	3.015	24085
5-Hydroxyindoleacetic acid	C10H9NO3	191.05824	2.196	2975
5'-Methylthioadenosine	C11H15N5O3S	297.08956	3.533	3425
Aconitic acid	C6H6O6	174.01644		3300
Adenosine-3'-monophosphate	C10H14N5O7P	347.06308	0.657	44736

Alanine	C3H7NO2	89.04768	0.343	11
alpha-Aminobutyric acid	C4H9NO2	103.06333		12
a-L-Rhamnose	C6H12O5	164.06847		1672
aminohippuric acid	C9H10N2O3	194.06914	1.400	3927
Aminoisobutyric acid	C4H9NO2	103.06333		480
Arabinose	C5H10O5	150.05282		311
Arginine	C6H14N4O2	174.11168	0.326	13
Asparagine	C4H8N2O3	132.05349	0.346	14
Aspartic Acid	C4H7NO4	133.03751	0.373	15
beta-Alanine	C3H7NO2	89.04768	0.333	36
Cadaverine	C5H14N2	102.11570	0.700	3236
Caffeine	C8H10N4O2	194.08038		1455
Citrulline	C6H13N3O3	175.09569	0.356	16
Creatine	C4H9N3O2	131.06948	0.364	7
Creatinine	C4H7N3O	113.05891		8
Cystathionine	C7H14N2O4S	222.06743	0.331	39
Cystine	C6H12N2O4S2	240.02385	0.336	17
Cytidine	C9H13N3O5	243.08552	0.385	3376
Deoxyribose	C5H10O4	134.05791		3258
D-Galactose	C6H12O6	180.06339	0.344	134
Dihydrocortisol	C21H32O5	364.22497		3173
DL-Ornithine	C5H12N2O2	132.08988	0.308	27
gamma-Aminobutryic acid	C4H9NO2	103.06333	0.324	279
glucose 6-phosphate	C6H13O9P	260.02972	0.401	145
Glutamic Acid	C5H9NO4	147.05316	0.349	19
Glutamine	C5H10N2O3	146.06914	0.342	18
Gly Pro	C7H12N2O3	172.08479		23672
Glycerol	C3H8O3	92.04734		105
Glycine	C2H5NO2	75.03203	0.371	20
Guanidine	CH5N3	59.04835		6342
Hippuric acid	C9H9NO3	179.05824	2.395	1301
Histidine	C6H9N3O2	155.06948		21
Hydroxylysine	C6H14N2O3	162.10044		47
Hypoxanthine	C5H4N4O	136.03851	0.612	83
Indoxylsulfuric acid	C8H7NO4S	213.00958		253
Isocitric acid	C6H8O7	192.02700	0.386	3328
Isoleucine	C6H13NO2	131.09463	0.504	23
Leucine	C6H13NO2	131.09463		24
Leucine Enkephalin	C28H37N5O7	555.26930	5.260	24069
Lysine	C6H14N2O2	146.10553	0.297	25
Malonic acid	C3H4O4	104.01096		3237
Mannitol	C6H14O6	182.07904	0.360	142
Methionine	C5H11NO2S	149.05105		26
Methylmalonic acid	C4H6O4	118.02661	0.605	3712

Methylsalicyluric acid	C10H11NO4	209.06881		5670
N(pai)-Methyl-L-histidine	C7H11N3O2	169.08513		3293
N,N'-butane-1,4-diyldiacetamide	C8H16N2O2	172.12118		
N,N'-pentane-1,5-diyldiacetamide	C9H18N2O2	186.13683		
N-[4-(3-acetamidopropylamino)butyl]acetamide	C11H23N3O2	229.17903		
N1,N12-Diacetylspermine	C14H30N4O2	286.23688		6525
N1-Acetylspermidine	C9H21N3O	187.16846		3323
N1-Acetylspermine	C12H28N4O	244.22631	0.700	3369
N8-Acetylspermidine	C9H21N3O	187.16846		24072
N-Acetylcadaverine	C7H16N2O	144.12626		6592
N-Acetyl-L-glutamic acid	C7H11NO5	189.06372	0.621	3325
N-Acetylputrescine	C6H14N2O	130.11061		3252
Niacin (Nicotinic acid)	C6H5NO2	123.03203	0.646	240
Niacinamide	C6H6N2O	122.04801	0.935	1497
p-Coumaric acid	C9H8O3	164.04734	4.022	307
Phenylalanine	C9H11NO2	165.07898		28
Proline	C5H9NO2	115.06333	0.366	29
Putrescine	C4H12N2	88.10005	0.279	3226
Pyroglutamic acid	C5H7NO3	129.04259	0.541	3251
Pyruvic acid	C3H4O3	88.01604	0.381	117
Riboflavin (Vitamin B2)	C17H20N4O6	376.13828	4.340	233
Sarcosine	C3H7NO2	89.04768	0.350	51
Serine	C3H7NO3	105.04259		30
Spermidine	C7H19N3	145.15790	0.261	254
Spermine	C10H26N4	202.21575	0.273	255
Succinic acid	C4H6O4	118.02661		114
Threonine	C4H9NO3	119.05824	0.343	32
Thymine	C5H6N2O2	126.04293		290
Trans-4-Hydroxy-L-proline	C5H9NO3	131.05824	0.349	257
Trimethylamine N-oxide	C3H9NO	75.06841		3773
Tryptophan	C11H12N2O2	204.08988	1.254	33
Tyrosine	C9H11NO3	181.07389		34
Uracil	C4H4N2O2	112.02728	0.439	258
Uric acid	C5H4N4O3	168.02834	0.585	88
Urocanic acid	C6H6N2O2	138.04293	0.381	298
Valine	C5H11NO2	117.07898	0.397	35
Tyrosine	C9H11NO3	181.07389		34
Uracil	C4H4N2O2	112.02728	0.439	258
Uric acid	C5H4N4O3	168.02834	0.585	88
Urocanic acid	C6H6N2O2	138.04293	0.381	298
Valine	C5H11NO2	117.07898	0.397	35
Xanthine	C5H4N4O2	152.03343	0.795	82

7.3 **Output data**

First step after data file loaded automatically searching was used - module Find compound by Molecular Feature. Molecular formula generation and compound identification were simultaneously with searching. As a source for searching and compound identification created database was used.

Settings: m/z from 60.0000 to 250.0000, peak with maximum height 600 counts

Ions allowed H^+, Na^+

Isotope model - common organic molecules, isotope grouping peak spacing tolerance 0,0025 m/z plus 5,0 ppm, limit assigned charge states to a maximum of 1

Absolute height to max 5000 counts

Filter mass list included masses from created database

Tab. 5: Number of founded compounds in each samples

F5 column	Number of Cpd	Hilic column	Number of Cpd
S 1	24	**S7**	**38**
S2	**40**	**MIX 2**	**23**
S3	1		
S4	31		
S5	31		
S6	26		
MIX 1	**34**		

S1-S3 plasma normal pH sample 1-3
S4-S6 plasma low pH sample 4-6
MIX standard mixture
S7 plasma normal pH sample

For detailed examination and identification of compounds was chosen just 1 sample from each column. Several measurements were found useless. That's why in case of HILIC column was measured just one sample.

For other steps of experiment were chosen Sample 2, Sample 7 and Mix 1 and 2.

Tab. 6: List of compounds - plasma S2, F5 column

	Name	Formula	Mass	m/z	RT	Height	Score	Ions
1	Glycerol	C3H8O3	92,0476	115,0368	0,572	384563	99,33	8
2	Serine	C3H7NO3	105,0427	106,05	0,594	5919	67,95	2
3	Glycine	C2H5NO2	75,0325	76,0398	0,61	11995	45,47	1
4	Pyroglutamic acid	C5H7NO3	129,0425	130,0498	0,613	163145	72,65	3
5	Glutamine	C5H10N2O3	146,0691	147,0764	0,615	238683	97,44	6
6	Threonine	C4H9NO3	119,0584	120,0657	0,634	88645	85,38	2
7	Valine	C5H11NO2	117,0793	118,0866	0,635	514725	87,16	2
8	Valine	C5H11NO2	117,0788	140,0681	0,647	119366	99,5	3
9	Citrulline	C6H13N3O3	175,0954	176,1026	0,667	9922	47,07	1
10	Sarcosine	C3H7NO2	89,0477	90,0549	0,668	25851	86,16	2
11	Proline	C5H9NO2	115,0642	116,0715	0,698	901758	81,93	2
12	a-L-Rhamnose	C6H12O5	164,0681	187,0573	0,709	11654	46,93	1
13	Proline	C5H9NO2	115,0641	138,0533	0,711	77468	43,84	1
14	a-L-Rhamnose	C6H12O5	164,068	187,0572	0,849	60485	83,83	2
15	Proline	C5H9NO2	115,0634	116,0707	0,878	13442	46,73	2
16	Histidine	C6H9N3O2	155,0693	156,0766	0,935	22307	47,35	2
17	Creatine	C4H9N3O2	131,0696	132,0769	0,968	36955	87,16	3
18	Deoxyribose	C5H10O4	134,058	157,0473	0,987	180331	99,46	3
19	N(pai)-Methyl-L-histidine	C7H11N3O2	169,0846	170,0919	1,048	7574	46,21	1
20	Valine	C5H11NO2	117,0793	118,0866	1,279	308035	99,18	3
21	Creatinine	C4H7N3O	113,059	114,0663	1,333	296604	85,63	4
22	Methionine	C5H11NO2S	149,051	150,0582	1,345	50786	98,72	3
23	Cytidine	C9H13N3O5	243,0869	244,0942	1,788	8465	39,94	1
24	Uric acid	C5H4N4O3	168,0298	169,0372	1,918	7464	38,33	2
25	Uric acid	C5H4N4O3	168,0296	169,0368	2,003	7217	40,02	1
26	Uric acid	C5H4N4O3	168,0295	169,0367	2,099	5305	41,19	1
27	Urocanic acid	C6H6N2O2	138,0423	139,0496	2,283	5676	45,3	1
28	Tyrosine	C9H11NO3	181,0733	182,0806	2,292	196600	96,34	3
29	p-Coumaric acid	C9H8O3	164,0467	165,054	2,293	46194	83,9	2
30	Leucine	C6H13NO2	131,0948	132,102	2,672	247485	99,82	4
31	N,N'-pentane-1,5-diyldiacetamide	C9H18N2O2	186,1361	209,1253	2,692	5984	73,96	2
32	D-Galactose	C6H12O6	180,0647	181,072	2,796	62338	77,42	2
33	Leucine	C6H13NO2	131,0949	132,1022	2,977	576045	99,55	4
34	Hippuric acid	C9H9NO3	179,0583	202,0476	3,061	16266	47,62	2
35	Caffeine	C8H10N4O2	194,0807	195,0879	3,769	106915	86,61	3
36	Phenylalanine	C9H11NO2	165,0789	166,0861	3,874	446357	99,2	3
37	a-L-Rhamnose	C6H12O5	164,0689	165,0762	5,208	7913	46,42	1
38	Tryptophan	C11H12N2O2	204,0899	205,0972	6,84	262747	87,53	4
39	Phenylalanine	C9H11NO2	165,0788	166,0861	8,993	9631	47,38	1
40	Uric acid	C5H4N4O3	168,0292	169,0365	16,924	8619	47,42	2

Tab. 7: List of compounds - Standard mixture MIX 1, F5 column

	Name	Formula	Mass	m/z	RT	Height	Score	Ions
1	N-Acetyl-L-glutamic acid	C7H11NO5	189,0629	190,0702	0,294	18806	59,2	2
2	Arabinose	C5H10O5	150,052	173,0412	0,541	5851	43,93	1
3	D-Galactose	C6H12O6	180,063	203,0522	0,548	29480	85,78	2
4	a-L-Rhamnose	C6H12O5	164,0681	187,0573	0,552	6492	46,96	1
5	Pyroglutamic acid	C5H7NO3	129,0424	152,0316	0,56	10953	76,62	2
6	Uracil	C4H4N2O2	112,0276	113,0349	0,56	51846	94,21	3
7	Aspartic Acid	C4H7NO4	133,0374	134,0447	0,562	12457	87,5	2
8	Glutamine	C5H10N2O3	146,0691	147,0764	0,564	11536	47,6	1
9	Pyroglutamic acid	C5H7NO3	129,0427	130,05	0,566	178591	99,31	3
10	Hippuric acid	C9H9NO3	179,058	202,0472	0,602	5807	47,38	1
11	Hippuric acid	C9H9NO3	179,0579	180,0652	0,609	225511	99,03	3
12	Glutamine	C5H10N2O3	146,069	147,0763	0,633	11432	47,46	1
13	Caffeine	C8H10N4O2	194,0799	195,0872	0,643	316326	98,22	3
14	p-Coumaric acid	C9H8O3	164,0467	165,054	0,644	37775	84,48	2
15	Niacinamide	C6H6N2O	122,0479	123,0552	0,702	201540	47,51	1
16	Niacin	C6H5NO2	123,0322	124,0395	0,73	202135	99,41	3
17	Aspartic Acid	C4H7NO4	133,0372	134,0445	0,775	102027	99,04	3
18	Pyroglutamic acid	C5H7NO3	129,0423	130,0495	0,789	42937	86,48	2
19	Glutamine	C5H10N2O3	146,0689	147,0762	0,79	222435	99,38	3
20	Proline	C5H9NO2	115,0634	116,0707	0,798	239999	87,58	2
21	Glycine	C2H5NO2	75,0324	76,0397	0,812	93017	86,47	2
22	Threonine	C4H9NO3	119,0584	120,0656	0,813	146607	78,21	2
23	Sarcosine	C3H7NO2	89,0481	90,0554	0,847	125779	86,47	2
24	Niacinamide	C6H6N2O	122,0478	123,0551	0,913	6777	47,44	1
25	Lysine	C6H14N2O2	146,1054	147,1127	0,99	112446	86,47	2
26	gamma-Aminobutryic acid	C4H9NO2	103,0637	104,071	1,001	43912	85,72	2
27	Cytidine	C9H13N3O5	243,0852	244,0924	1,151	49837	98,07	3
28	Methionine	C5H11NO2S	149,0509	150,0582	1,209	114780	47,56	1
29	Arabinose	C5H10O5	150,0533	151,0606	1,24	7267	46,09	1
30	Tyrosine	C9H11NO3	181,0735	182,0808	1,71	46706	84,27	2
31	Leucine	C6H13NO2	131,0947	132,1019	2,115	163544	99,61	3
32	Phenylalanine	C9H11NO2	165,0787	166,0859	3,105	132952	99	3
33	Tryptophan	C11H12N2O2	204,0895	205,0968	5,778	123306	99,28	4
34	Valine	C5H11NO2	117,0791	118,0864	15,798	44731	86,24	2

Tab. 8: List of compounds - plasma S7, HILIC column

	Name	Formula	Mass	m/z	RT	Height	Score	Ions
1	Caffeine	C8H10N4O2	194,0805	195,0878	0,601	105858	97,65	4
2	Hippuric acid	C9H9NO3	179,0581	180,0654	0,985	15911	47,53	1
3	Hippuric acid	C9H9NO3	179,0579	202,0471	1,002	6749	46,87	1
4	Urocanic acid	C6H6N2O2	138,0425	139,0498	1,067	5611	46,51	1
5	Glycerol	C3H8O3	92,0475	115,0367	1,136	9150	47,39	1
6	N-Acetylcadaverine	C7H16N2O	144,1264	145,1337	1,23	6379	47,47	1
7	Creatinine	C4H7N3O	113,0591	114,0664	1,589	12950	47,43	1
8	N-Acetylcadaverine	C7H16N2O	144,1263	145,1336	1,84	112434	99,16	3
9	N-Acetylputrescine	C6H14N2O	130,1104	131,1177	2,023	14591	47,34	1
10	a-L-Rhamnose	C6H12O5	164,0678	187,057	2,069	35041	85	2
11	Phenylalanine	C9H11NO2	165,0786	188,0678	2,575	5734	46,71	1
12	Pyroglutamic acid	C5H7NO3	129,0426	130,0498	3,214	20494	83,2	2
13	Phenylalanine	C9H11NO2	165,0787	166,086	3,391	88471	97,82	3
14	Tryptophan	C11H12N2O2	204,0899	227,0791	3,633	11041	82,57	2
15	Tryptophan	C11H12N2O2	204,0897	205,097	3,637	48923	92,98	3
16	Valine	C5H11NO2	117,0789	140,0681	3,817	8640	68,63	2
17	Methionine	C5H11NO2S	149,0512	150,0584	4,137	13388	47,56	2
18	Uric acid	C5H4N4O3	168,0281	169,0354	4,316	9224	77,72	3
19	Valine	C5H11NO2	117,0791	118,0864	4,38	123625	98,9	3
20	Proline	C5H9NO2	115,0638	116,0711	4,382	425339	87,02	5
21	Valine	C5H11NO2	117,0787	140,0679	4,611	11745	77,21	2
22	Tyrosine	C9H11NO3	181,0735	182,0807	4,712	28401	86,04	2
23	Creatine	C4H9N3O2	131,0695	132,0768	5,233	128956	99,06	4
24	Creatinine	C4H7N3O	113,059	136,0483	5,234	5070	47,51	1
25	Sarcosine	C3H7NO2	89,0479	90,0552	5,303	32771	87,89	3
26	Threonine	C4H9NO3	119,0583	120,0656	5,478	13103	47,61	2
27	Glycine	C2H5NO2	75,0323	76,0395	5,624	5333	47,21	2
28	Pyroglutamic acid	C5H7NO3	129,0423	130,0496	5,867	35739	83,62	3
29	Glutamine	C5H10N2O3	146,0689	147,0762	5,868	176455	98,21	5
30	Asparagine	C4H8N2O3	132,0536	133,0609	5,988	6492	47,55	2
31	Glutamic Acid	C5H9NO4	147,0529	148,0601	6,071	6790	47,12	1
32	N(pai)-Methyl-L-histidine	C7H11N3O2	169,0847	170,092	6,13	12217	76,7	2
33	Citrulline	C6H13N3O3	175,0952	176,1025	6,151	8122	80,37	2
34	Histidine	C6H9N3O2	155,0693	156,0766	6,661	156204	98,63	4
35	N(pai)-Methyl-L-histidine	C7H11N3O2	169,0848	170,0921	6,742	5067	47,15	1
36	Arginine	C6H14N4O2	174,1112	175,1184	6,823	37077	79,56	2
37	Lysine	C6H14N2O2	146,1052	147,1125	6,979	19623	77,58	3
38	Proline	C5H9NO2	115,0637	116,071	7,048	12091	75,13	2

Tab. 9: List of compounds - Standard mixture MIX 2, HILIC column

	Name	Formula	Mass	m/z	RT	Height	Score	Ions
1	p-Coumaric acid	C9H8O3	164,0474	165,0547	0,616	39896	95,51	5
2	Uracil	C4H4N2O2	112,0278	113,0351	0,86	150406	86,02	2
3	Hippuric acid	C9H9NO3	179,0582	180,0655	0,982	354394	99,75	3
4	Caffeine	C8H10N4O2	194,0797	195,087	1,117	6001	70,06	2
5	Deoxyribose	C5H10O4	134,0578	157,047	1,118	82117	99,62	3
6	Niacin	C6H5NO2	123,0321	146,0213	1,239	18942	77,42	2
7	Cytidine	C9H13N3O5	243,0853	244,0925	2,878	180754	98,76	3
8	Pyroglutamic acid	C5H7NO3	129,0427	130,05	3,197	292457	99,02	6
9	Tryptophan	C11H12N2O2	204,0898	205,0971	3,618	390664	99,9	3
10	Methionine	C5H11NO2S	149,0514	150,0587	4,114	445268	99,03	7
11	gamma-Aminobutryic acid	C4H9NO2	103,0638	104,0711	4,26	58780	86,65	6
12	D-Galactose	C6H12O6	180,063	203,0522	4,274	36180	85,85	2
13	p-Coumaric acid	C9H8O3	164,0474	165,0547	4,701	7863	47,6	1
14	Tyrosine	C9H11NO3	181,0736	182,0809	4,703	96203	98,53	5
15	D-Galactose	C6H12O6	180,063	203,0522	4,898	51340	95,92	3
16	Sarcosine	C3H7NO2	89,0482	90,0555	5,294	161733	86,91	4
17	Threonine	C4H9NO3	119,0582	120,0655	5,488	101766	47,62	1
18	Glutamine	C5H10N2O3	146,0697	147,077	5,857	442269	98,85	6
19	Pyroglutamic acid	C5H7NO3	129,0427	130,0499	5,858	107939	87,45	3
20	Aspartic Acid	C4H7NO4	133,0375	134,0448	6,499	82531	98,25	3
21	Niacin (Nicotinic acid)	C6H5NO2	123,0321	124,0394	6,609	8468	47,6	1
22	Arginine	C6H14N4O2	174,1111	175,1183	6,802	5705	70,14	2
23	Proline	C5H9NO2	115,0638	116,0711	7,025	8160	46,12	1

PART 3 – Results and discussion

8 Identification methods

Main method for identification was compare of peaks, primarily retention time, then also shape and height of peak, mass and m/z. For identification was used simple comparing of peaks found by automatically or manually searching.

8.1 Peaks comparisone

Evaluation was based on comparison of peak of compound in plasma sample and peak of the potentially same peak of compound in standard sample with assigned name, formula and mass. In case that by automatically searching was found in one sample more compounds identified with same name, formula and mass, but with different m/z, the peaks of compounds were often same, because in setting were allow search compounds in form $[M+H]^+$ and $[M+Na]^+$. In case the peak was not found by automatically searching the manual searching method was used. It was used also for control of automatically searching.

8.2 Manual searching

Two searching modules of manual searching were used, module Find compounds by formula and Define a chromatogram.

Settings - Find compounds by formula:

- Formula or database was placed as source for formula, mass tolerance 5ppm (or higher if needed), expansion of values for chromatogram extraction possible m/z value 100 ppm, enable ions H^+, Na^+, charge state range 1, type of chromatogram EIC. Result was a peak matched on concrete formula.

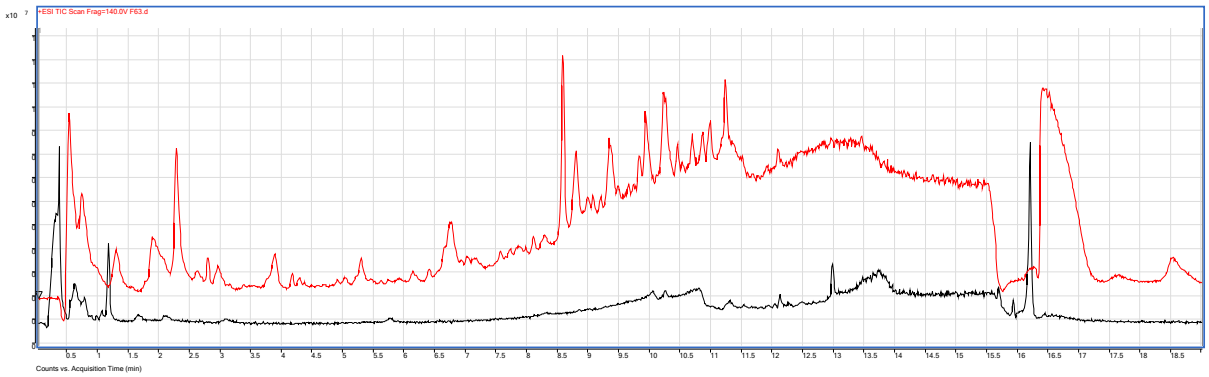
Settings - Define a chromatogram:

- Set a concrete m/z value and m/z expansion (5 or higher if needed) for chromatogram in ppm, type of chromatogram set EIC, type of polarity set positive.

8.3 **F5 column**

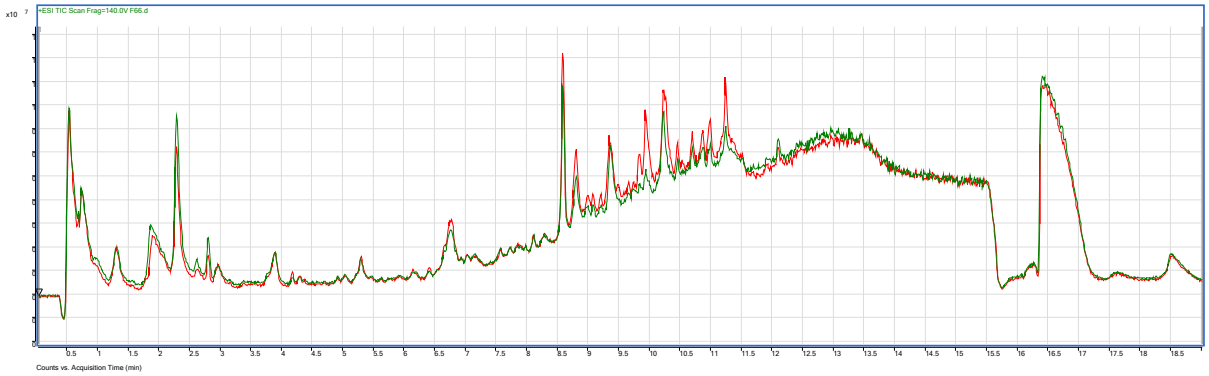
There are shown whole chromatograms of plasma sample S2 and standard mixture sample MIX1 measured on F5 column. Methods for identification are shown on few examples of selected compounds.

Fig. 4: Whole chromatogram of plasma sample S2 and standard mixture sample MIX 1



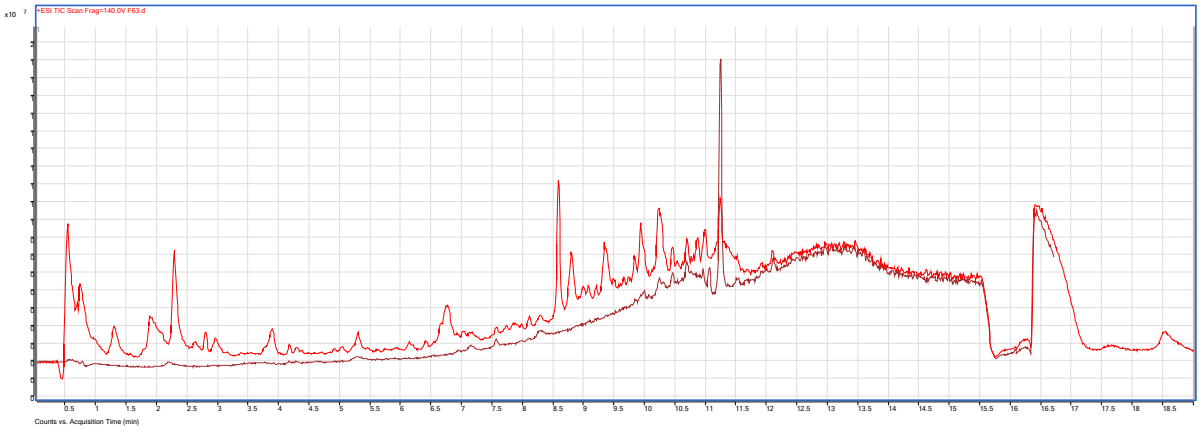
Red color – plasma sample S2, black color – standard mixture sample MIX1

Fig. 5: Whole chromatogram of plasma sample S2 (normal pH) and S5 (low pH)



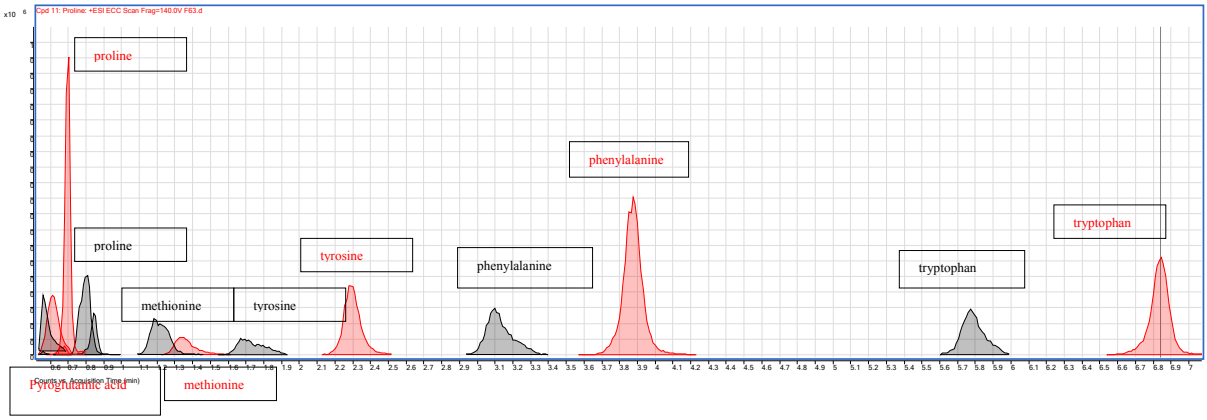
Red color – plasma sample S2 normal pH, green color – plasma sample S5 low pH

Fig. 6: Whole chromatogram of plasma sample S2 and control blank sample



Red color – plasma sample S2, dark red color – control blank sample

Fig. 7: Schema of same identified compounds selected in plasma sample S2 and standard mixture sample MIX1



Red color – plasma sample S2, black color - standard mixture sample MIX1

From the whole chromatogram of plasma sample S2 and standard mixture sample MIX1 is visible that the surveyed compound are in the beginning of elution.

From whole chromatogram of plasma sample S2 prepared in normal pH and S5 prepared in low pH are visible minimum differences between samples. pH of samples is not important.

Control with blank sample shown that there are no important impurities in the mobile phase or in the HPLC.

Schema shows a position of compounds in first 7 minutes of elution. Peaks of the same identified compounds from two measured samples have not same retention time.

8.3.1 **Methionine**

Number of Cpd	Name	Formula	Mass	m/z	RT	Vol	Score
Plasma - 22	Methionine	C5H11NO2S	149,0510	150,0582	1,345	424965	98,72
Mix - 28	Methionine	C5H11NO2S	149,0509	150,0582	1,209	817855	47,56

Fig. 8: Peak of plasma sample S2 Cpd 22 and standard mixture sample MIX 1 Cpd 28

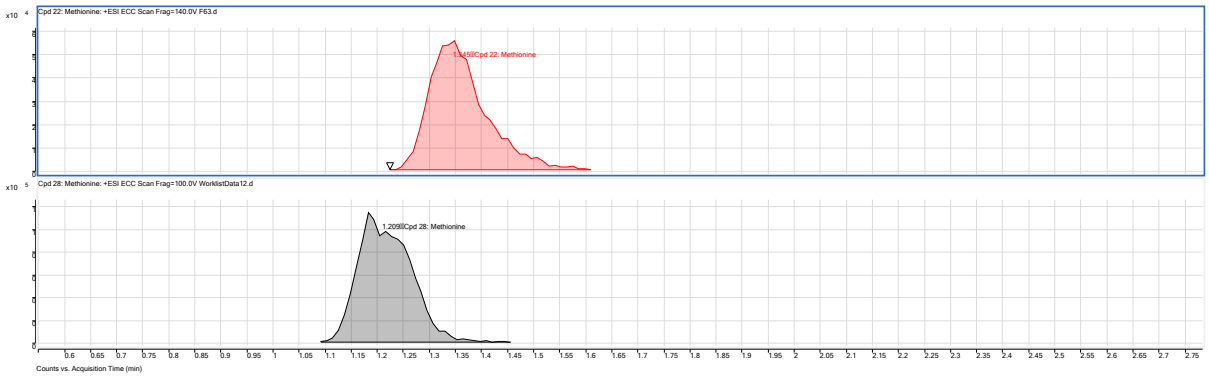
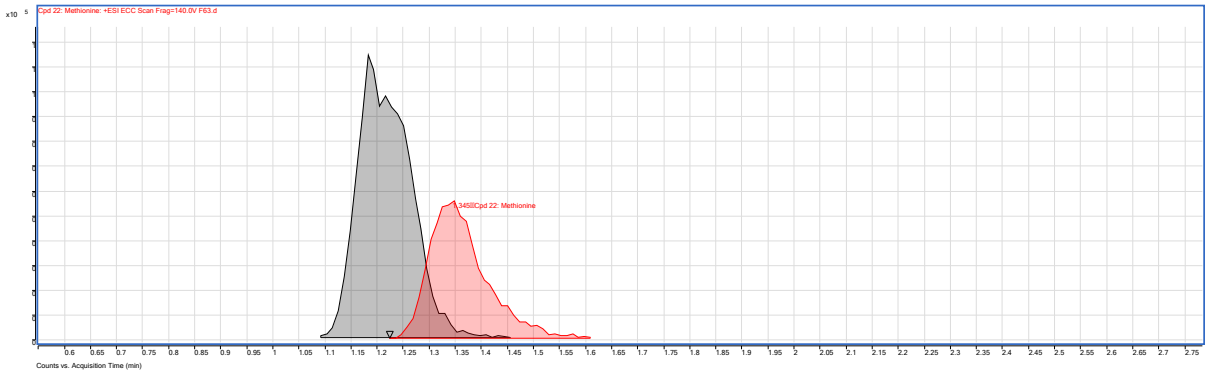


Fig. 9: Overlap peaks of plasma sample S2 Cpd 22 and standard mixture sample MIX 1 Cpd 28

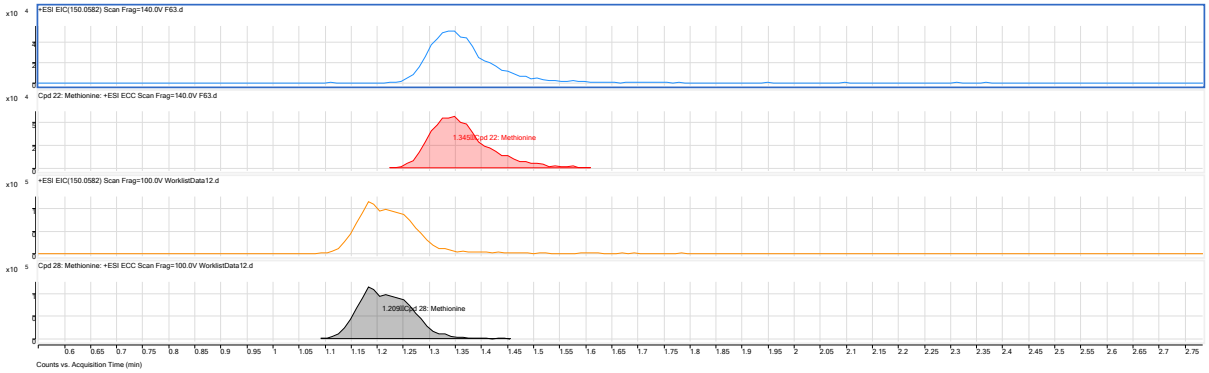


Automatically searching found one peak identified as methionine in plasma sample S2 (Cpd 22, red color) and one peak with same identification in standard mixture sample MIX1 (Cpd 28, black color).

Overlap chromatogram shows that concentration of Cpd 28 in standard mixture sample MIX1 is higher.

Peaks have different retention time, they are no strict overlap. This is affected by different conditions of measuring samples.

Fig. 10: Peak of plasma sample S2 Cpd 22 and standard mixture sample MIX 1 Cpd 28 and competent control peaks generated by manual searching



Control by manual searching (module Define a chromatogram) of current peaks was used. Blue peak according to red peak of Cpd 22, plasma sample S2 and orange peak according to black peak of Cpd 28, standard mixture sample MIX1.

No other peaks were found by manual searching.

8.3.2 **Proline**

Number	Name	Formula	Mass	m/z	RT	Vol	Score
Plasma - 11	Proline	C5H9NO2	115,0642	116,0715	0,698	2123239	81,93
Plasma - 13	Proline	C5H9NO2	115,0641	138,0533	0,711	164232	43,84
Plasma - 15	Proline	C5H9NO2	115,0634	116,0707	0,878	180284	46,73
Mix - 20	Proline	C5H9NO2	115,0634	116,0707	0,798	1120814	87,58

Fig. 11: Peaks of plasma sample S2 Cpd 11, Cpd 13, Cpd 15

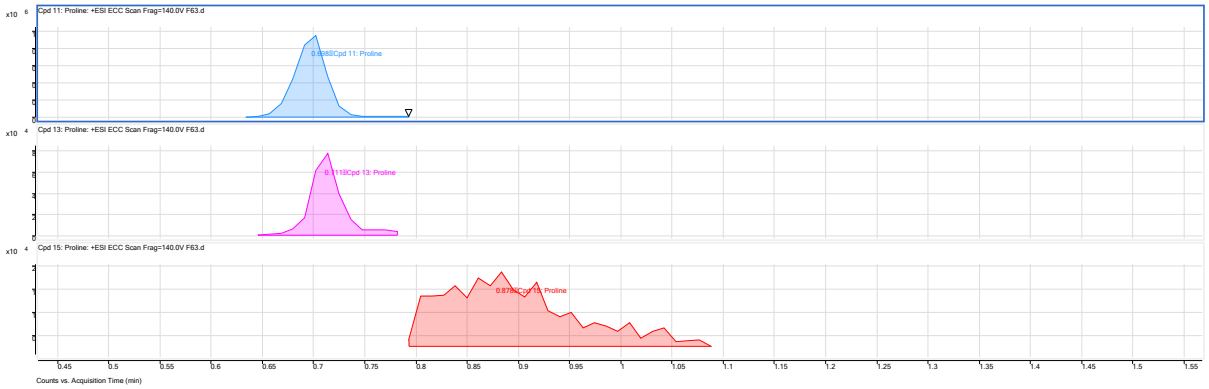
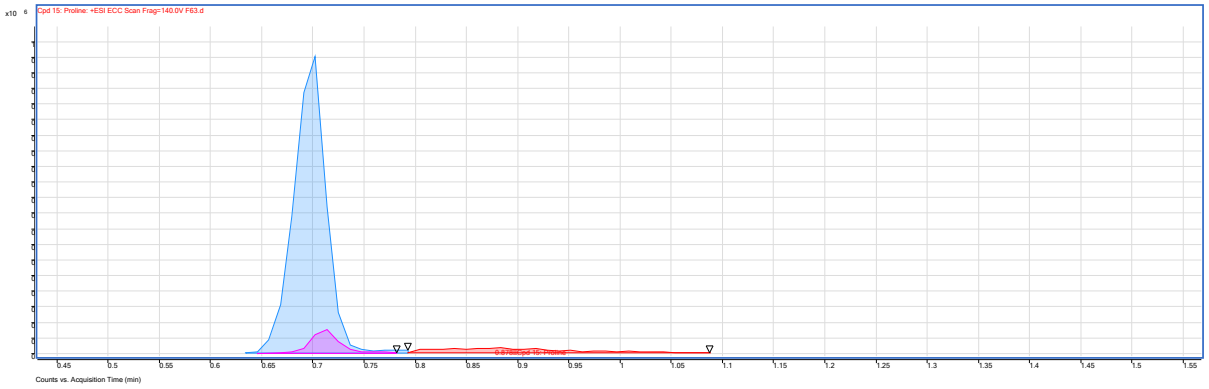


Fig. 12: Overlap peaks of plasma sample S2 Cpd 11, Cpd 13, Cpd 15



Automatically searching found three peaks identified as proline in plasma sample S2 (Cpd 11 blue color, Cpd 13 pink color, Cpd 15 red color) and just one peak with same identification in standard mixture sample MIX1 (Cpd 20, violet color, Fig.13)

Cpd 11 and 13 in plasma sample S2 are differ in m/z value. Cpd 13 is in form $[M+Na]^+$ and Cpd 11 $[M+H]^+$.

Overlap chromatogram shows that concentration of Cpd 11 in plasma sample S2 is much higher.

Peak of Cpd 15 in plasma sample S2 has different retention time than Cpd 11 and 13 and bad shape of peak.

Fig. 13:Overlap peaks of plasma sample S2 Cpd 11, Cpd 13, Cpd 15 and standard mixture sample MIX1 Cpd 20

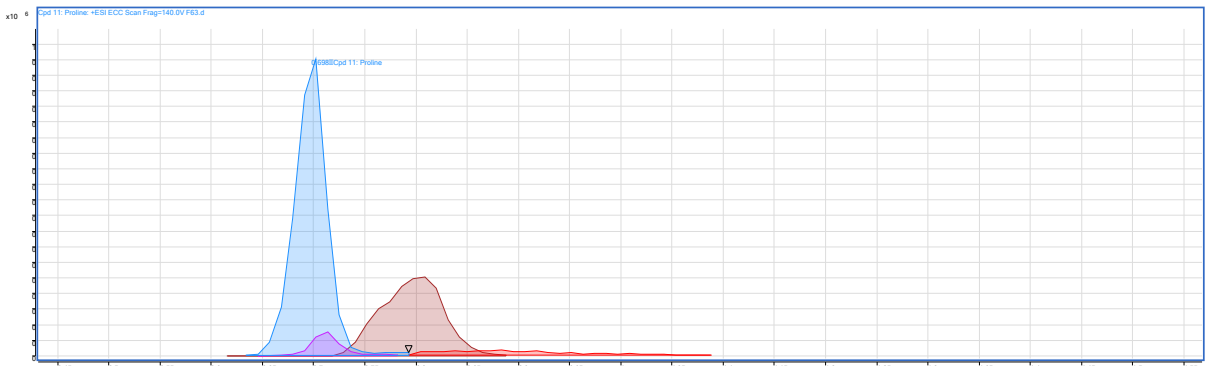
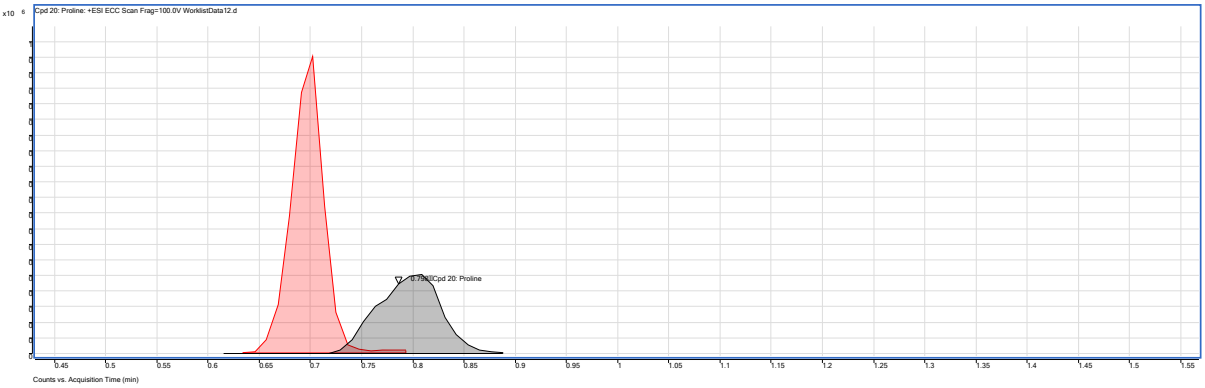


Fig. 14: Overlap chromatogram of plasma sample S2 Cpd 11 and standard mixture sample MIX1 Cpd 20



From 3 possibilities in plasma sample S2 Cpd 11 (red color in Fig.14) was selected and compared with standard mixture sample MIX1 Cpd 20 (black color in Fig.14). Peaks have different retention time affected by different conditions of measuring samples.

8.3.3 **Pyroglutamic acid**

Number	Name	Formula	Mass	m/z	RT	Vol	Score
Plasma - 4	Pyroglutamic acid	C5H7NO3	129,0425	130,0498	0,613	950223	72,65
Mix - 5	Pyroglutamic acid	C5H7NO3	129,0424	152,0316	0,56	18676	76,62
Mix - 9	Pyroglutamic acid	C5H7NO3	129,0427	130,05	0,566	675158	99,31
Mix - 18	Pyroglutamic acid	C5H7NO3	129,0423	130,0495	0,789	158921	86,48

Fig. 15: Peak of plasma sample S2 Cpd 4

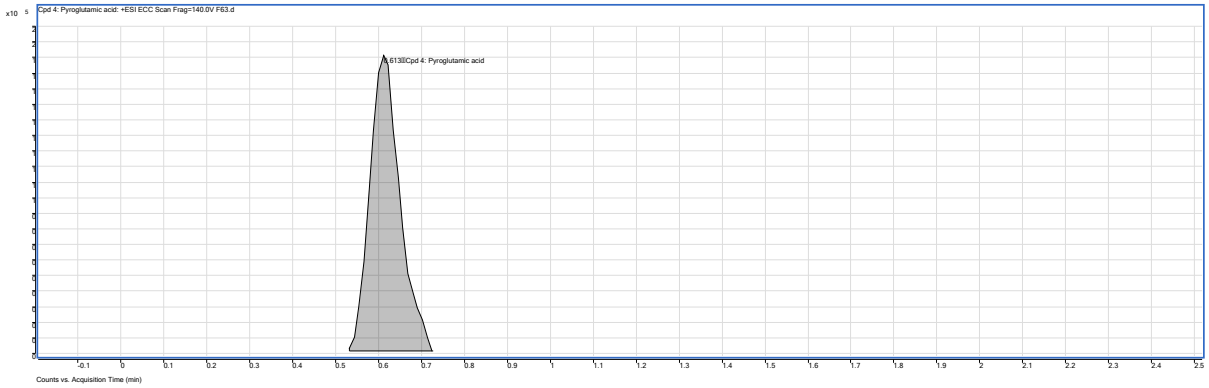
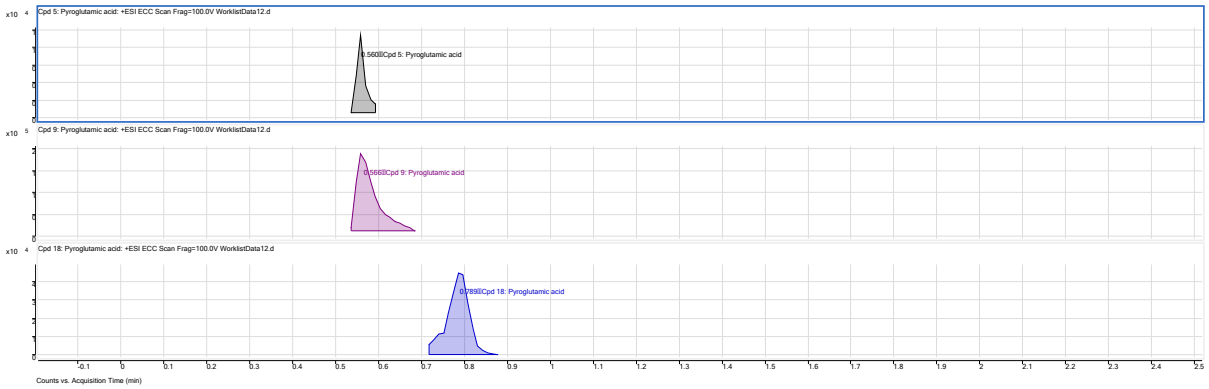


Fig. 16: Peaks of standard mixture sample MIX1 Cpd 5, Cpd 9, Cpd 18



Automatically s earching f ound one peak i dentified a s pyr oglutamic a cid i n pl asma sample S2 (Cpd 4, black c olor) and three peaks with same id entification in standard mixture sample MIX1 (Cpd 5 grey color, Cpd 9 violet color, Cpd 18 blue color)

Cpd 5 in standard mixture sample MIX1 is differ in m/z value (it is in form $[M+ Na]^{+}$) and was excluded.

Fig. 17: Overlap chromatogram of plasma sample Cpd 4 and standard mixture sample MIX1 Cpd 9, Cpd 18

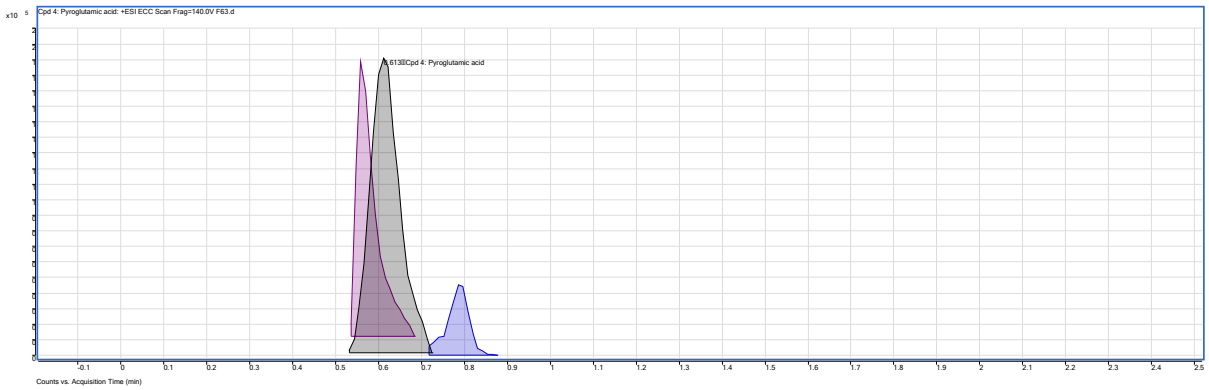
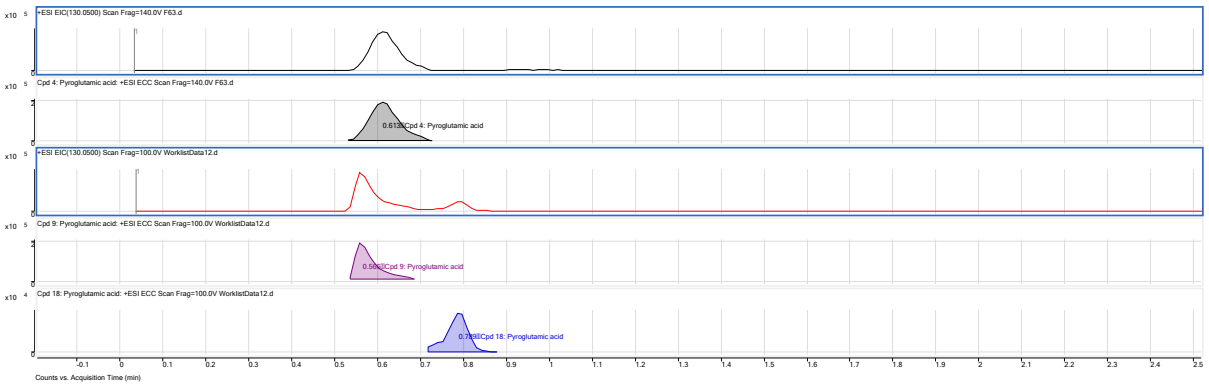


Fig. 18: Peak of plasma sample S2 Cpd 4 and standard mixture sample MIX 1 Cpd 9, Cpd 18 and competent control peaks generated by manual searching



Overlap chromatogram shows that concentration of Cpd 18 in standard mixture sample MIX1 is low.

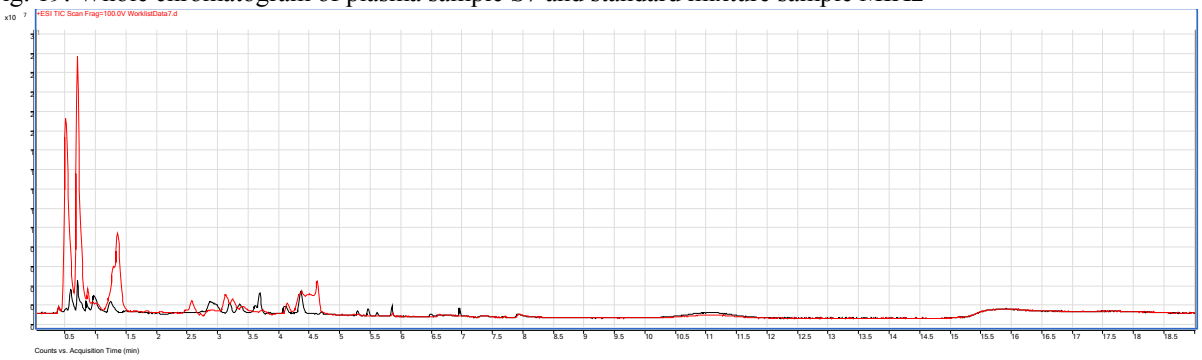
Control by manual searching (module Define a chromatogram) of current peaks was used. Black line according to black peak of Cpd 4, plasma sample S2 and red line according to violet and blue peaks of Cpd 9 and 18, standard mixture sample MIX1.

According to concentration and score value standard mixture sample MIX1 Cpd 18 was excluded.

8.4 HILIC column

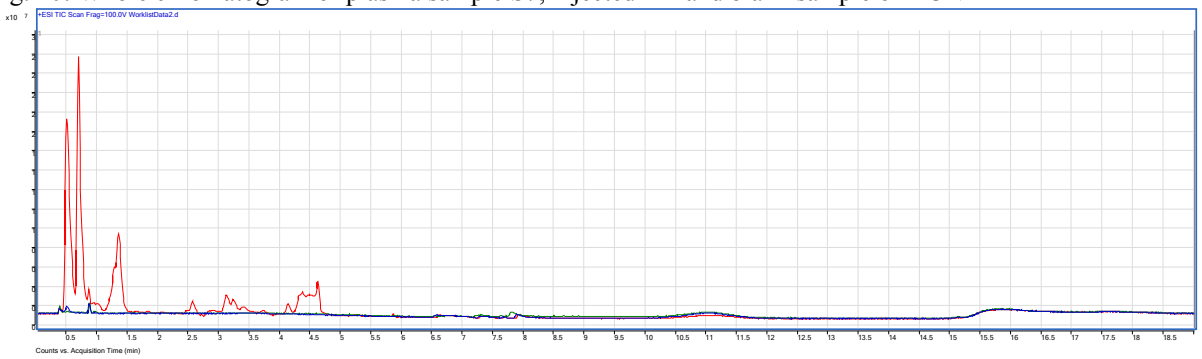
There are shown whole chromatograms of plasma sample S7 and standard mixture sample MIX2 measured on HILIC column. Methods for identification are shown on few examples of selected compounds.

Fig. 19: Whole chromatogram of plasma sample S7 and standard mixture sample MIX2



Red color – plasma sample S7, black color – standard mixture sample MIX2

Fig. 20: Whole chromatogram of plasma sample S7, injected Air and blank sample of ACN

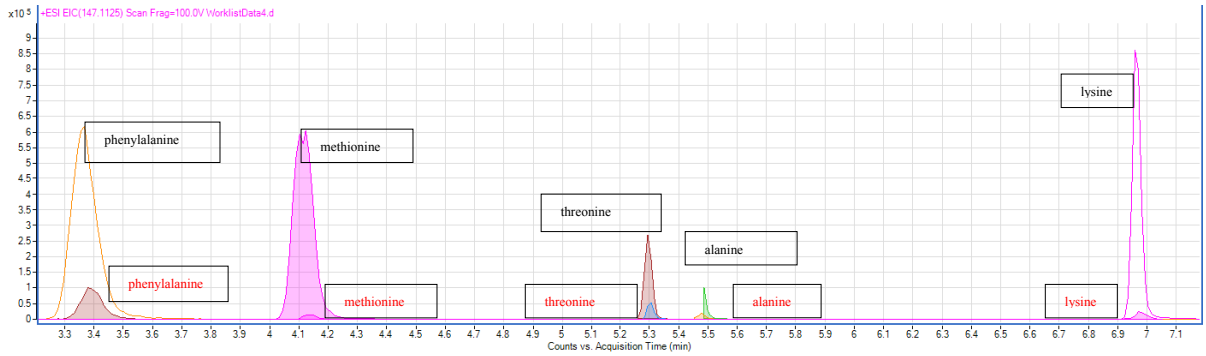


Red color – plasma sample S7, blue color – Air, green color - blank sample of ACN

From the whole chromatogram of plasma sample S7 and standard mixture sample MIX2 is visible that the surveyed compound are in the beginning of elution.

Control with blank sample shows that there are no important impurities in the mobile phase or in HPLC

Fig. 21: Schema of same identified compounds selected in plasma sample S7 and standard mixture sample MIX2



Red color – plasma sample S7, black color - standard mixture sample MIX2

Schema shows a position of compounds in firts 7 minutes of elution. Peaks of same identified compounds from two measured samples have almost same retention time and they are overlap.

8.4.1 **Tryptophan**

Number	Name	Formula	Score	Mass	m/z	RT	Vol
Plasma - 14	Tryptophan	C11H12N2O2	82.57	204.0899	227.0791	3.633	47222
Plasma - 15	Tryptophan	C11H12N2O2	92.98	204.0897	205.097	3.637	249293
Mix - 9	Tryptophan	C11H12N2O2	99.90	204,0898	205,0971	3,618	2145815

Fig. 22: Overlap chromatogram of plasma sample S7 Cpd 14, Cpd 15

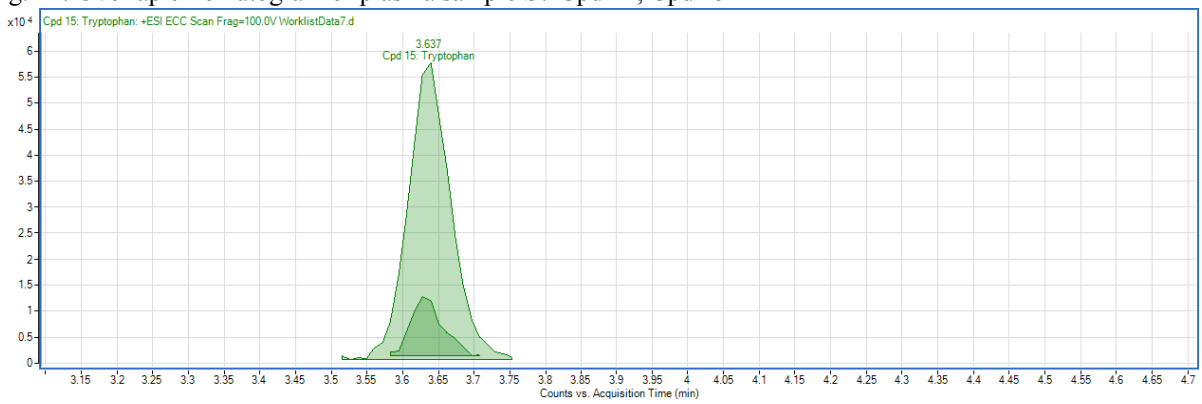
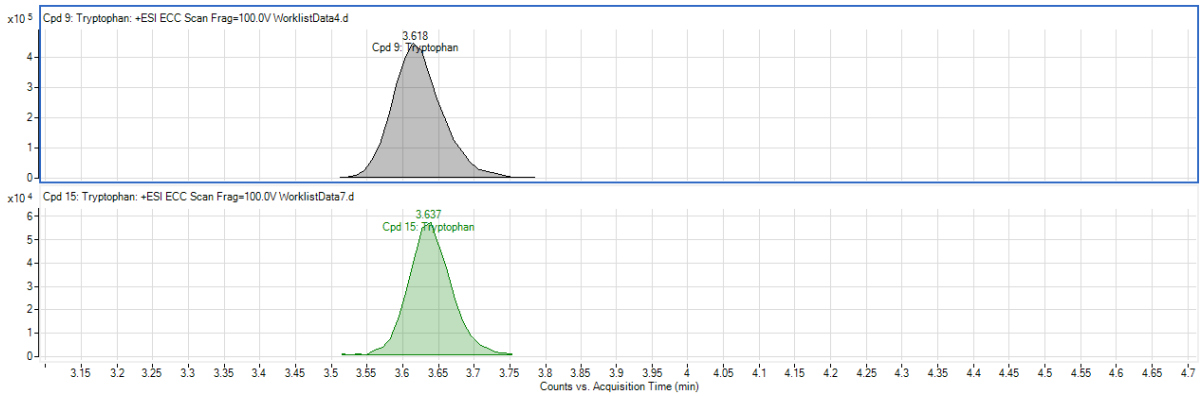


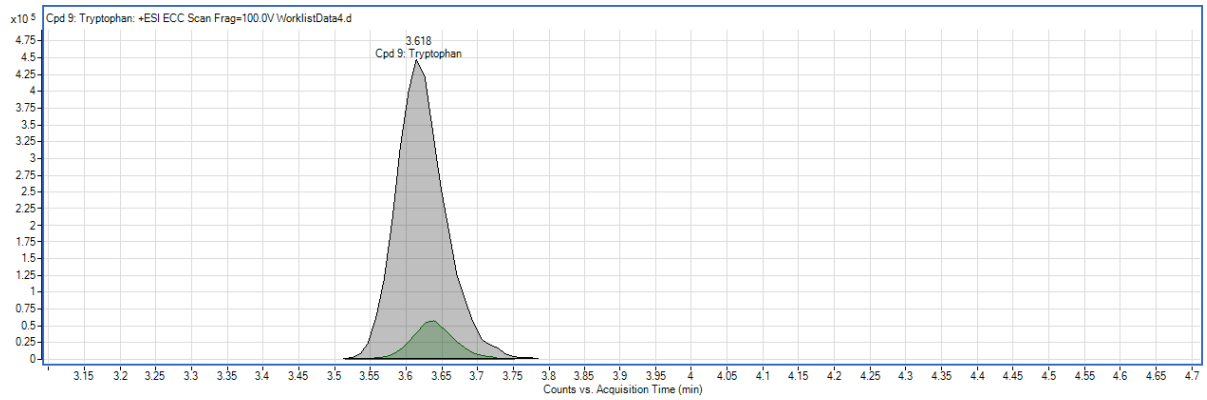
Fig. 23: Peaks of standard mixture sample MIX2 Cpd 9 and plasma sample S7 Cpd 15



Automatically searching found two peaks identified as tryptophan in plasma sample S7 (Cpd 14, dark green color and Cpd 15 green color) and one peak with same identification in standard mixture sample MIX2 (Cpd 9, black color).

Cpd 14 in plasma sample S7 is differ in m/z value (it is in form $[M+ Na]^+$), has lower concentration and was excluded.

Fig. 24: Overlap chromatogram of plasma sample S7 Cpd 15 and standard mixture sample MIX2 Cpd 9



Overlap chromatogram of plasma sample S7 Cpd 15 (green color) and standard mixture sample MIX2 Cpd 9 (black color) shows that concentration of Cpd 9 is higher. Peaks have almost same retention time and are overlap.

8.4.2 **Caffeine**

Number	Name	Formula	Score	Mass	m/z	RT	Vol
Plasma - 1	Caffeine	C8H10N4O2	97.65	194.0805	195.0878	0.601	396663
Mix - 4	Caffeine	C8H10N4O2	70,06	194,0797	195,087	1,117	21812

Fig. 25: Peaks of standard mixture sample MIX2 Cpd 4 and plasma sample S7 Cpd 1

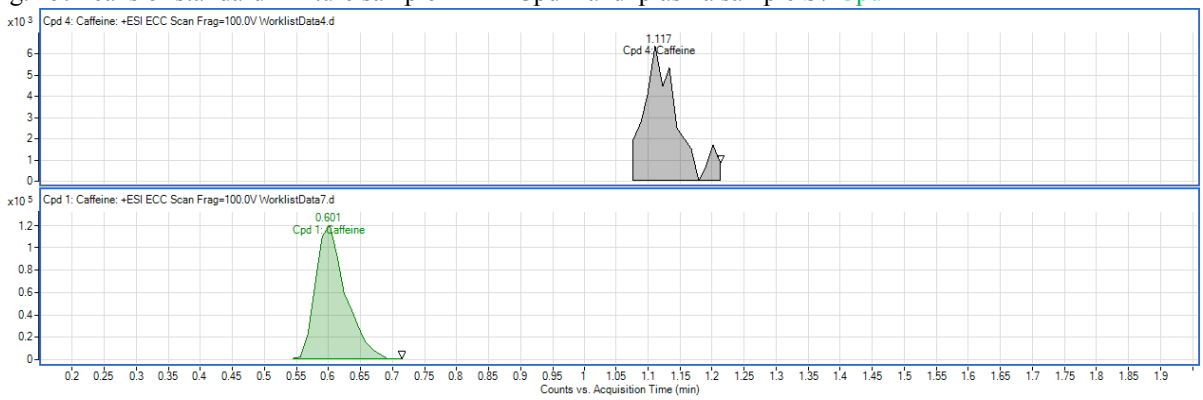


Fig. 26: Overlap chromatogram of standard mixture sample MIX2 Cpd 4 and plasma sample S7 Cpd 1

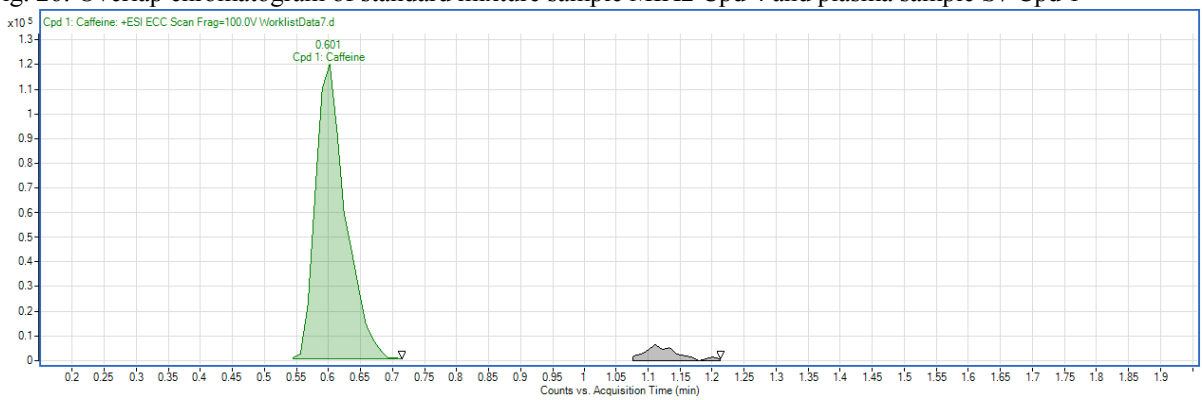


Fig. 27: Peak of standard mixture sample MIX2 Cpd 4 and competent two control peaks generated by manual searching

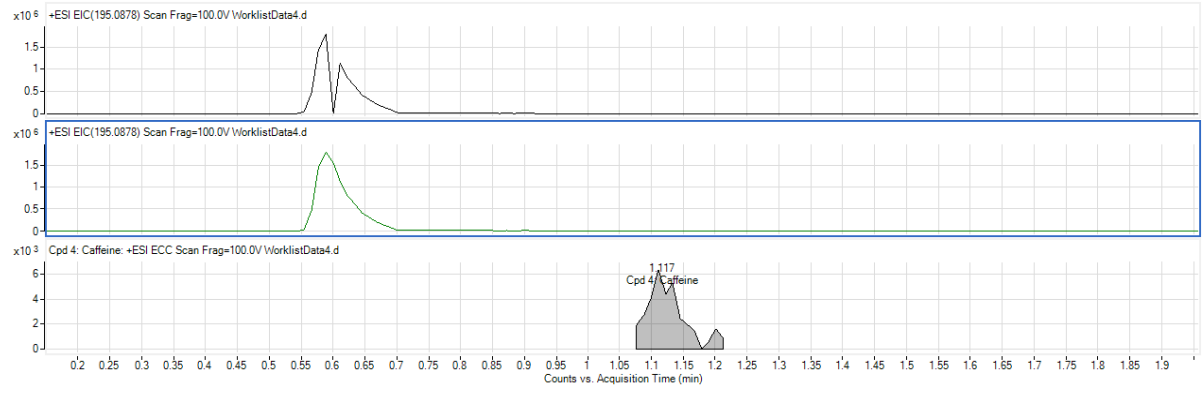


Fig. 28: Peak of plasma sample S7 Cpd 1, control peak of Cpd 1 generated by manual searching and control peak of standard mixture sample MIX2 Cpd 4

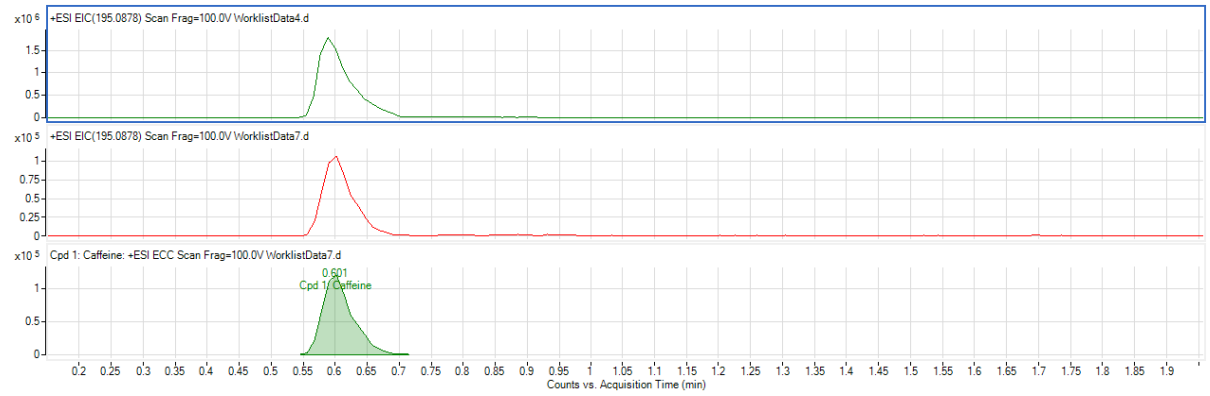
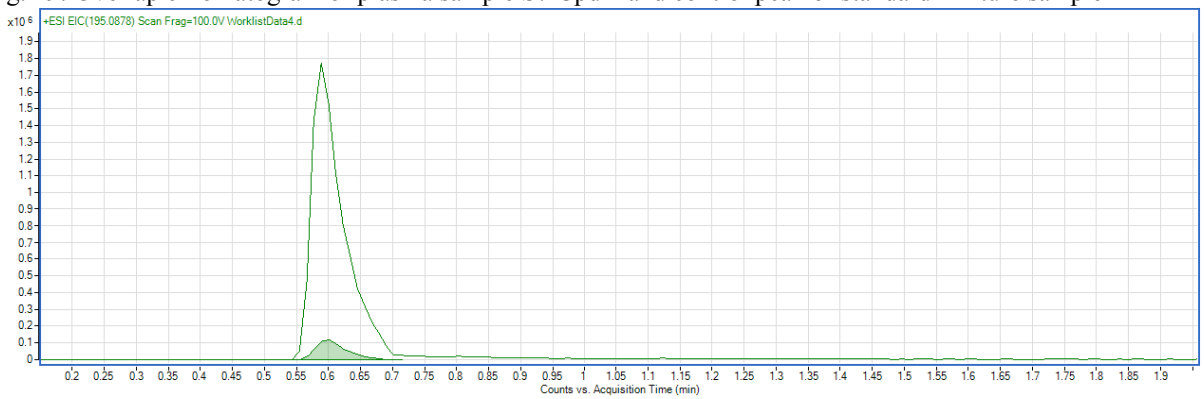


Fig. 29: Overlap chromatogram of plasma sample S7 Cpd 1 and control peak of standard mixture sample MIX2



Automatically searching found one peak identified as caffeine in plasma sample S7 (Cpd 1, green color) and one peak with same identification in standard mixture sample MIX2 (Cpd 4, black color).

The shape of peak of Cpd 4 in standard mixture sample MIX2 is bad, retention time different and the concentration very low. The control by manual searching (module Define a chromatogram) was used. First control peak (black line) of standard mixture sample MIX2, Cpd 4, generated by module Define a chromatogram with peak spacing tolerance 10 ppm, was sliced, second control peak (green line) was generated with peak spacing tolerance 20 ppm.

Second control peak according to peak and control peak (red line) of plasma sample S7 Cpd 1. Concentration of Cpd 4 (expressed by second control peak of standard mixture sample MIX2, Cpd 4) is very high.

8.4.3 **Phenylalanine**

Number	Name	Formula	Score	Mass	m/z	RT	Vol
Plasma - 11	Phenylalanine	C9H11NO2	46.71	165.0786	188.0678	2.575	24642
Plasma - 13	Phenylalanine	C9H11NO2	97.82	165.0787	166.086	3.391	542033
Mix - --	----------	----------	----------	----------	----------	----------	----------

Fig. 30: Peaks of plasma sample S7 Cpd 11 and Cpd 13

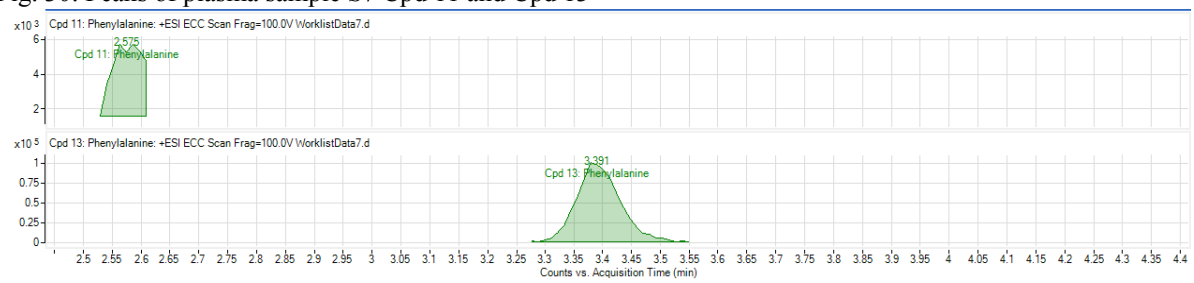
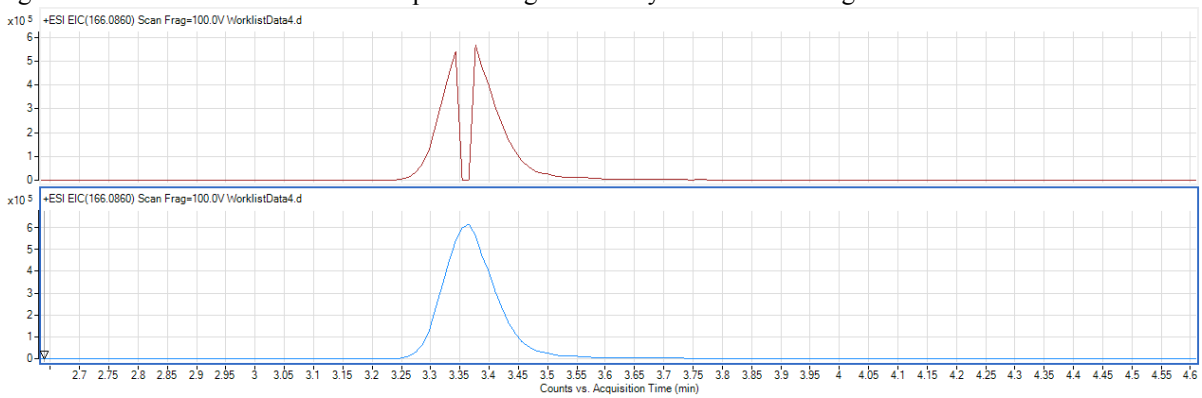


Fig. 31: Peaks of standard mixture sample MIX2 generated by manual searching

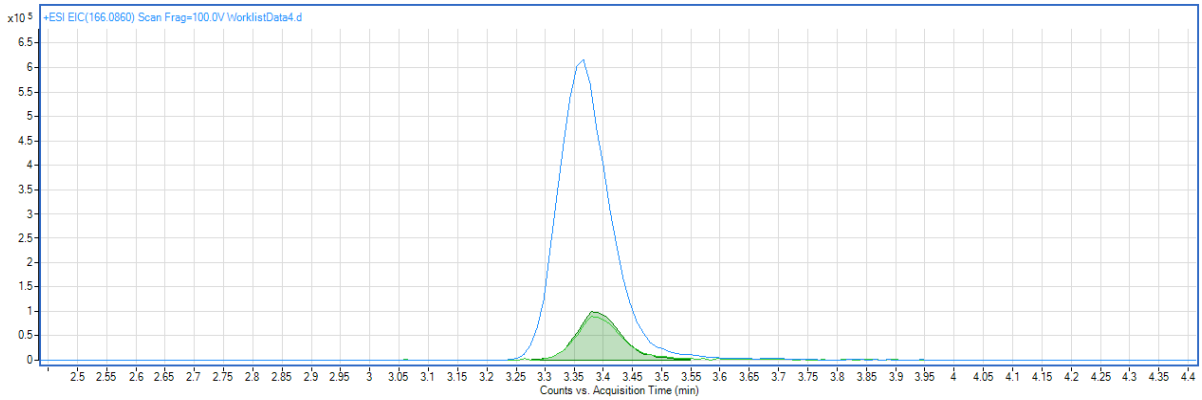


Automatically s earching f ound t wo peaks i dentified as p henylalanine in plasma sample S 7 (Cpd 11 a nd C pd 13, green c olor) and none peak w ith s ame i dentification in standard mixture sample MIX2.

Cpd 11 of plasma s ample S 7 w as ex cluded b ecause t he shape of pe ak was ba d a nd retention time was different.

For standard mixture sample MIX2 was peak generated manually by module Define a chromatogram - first peak (red line) with peak spacing tolerance 10 pp m was sliced, second peak (blue line) with peak spacing tolerance 20 ppm.

Fig. 32: Overlap chromatogram of plasma sample S7 Cpd 13 and manuallly generated peak of standard mixture sample MIX2

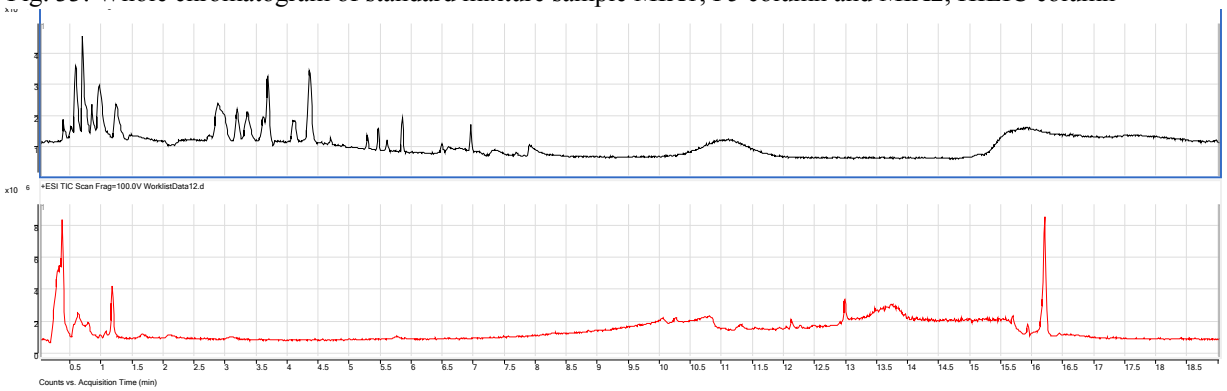


Second peak of standard mixture sample MIX2 generated by manual searching was overlap with peak of plasma sample S7, Cpd 13.

8.5 **Comparison of the columns**

Characterization of F 5 column is particle size 2,7 µm and size of the column 5 cm x 2,1 mm, of HILIC column is particle size 1,7 µm and size of the column 2,1x100mm. HILIC column is two times shorter.

Fig. 33: Whole chromatogram of standard mixture sample MIX1, F5 column and MIX2, HILIC column



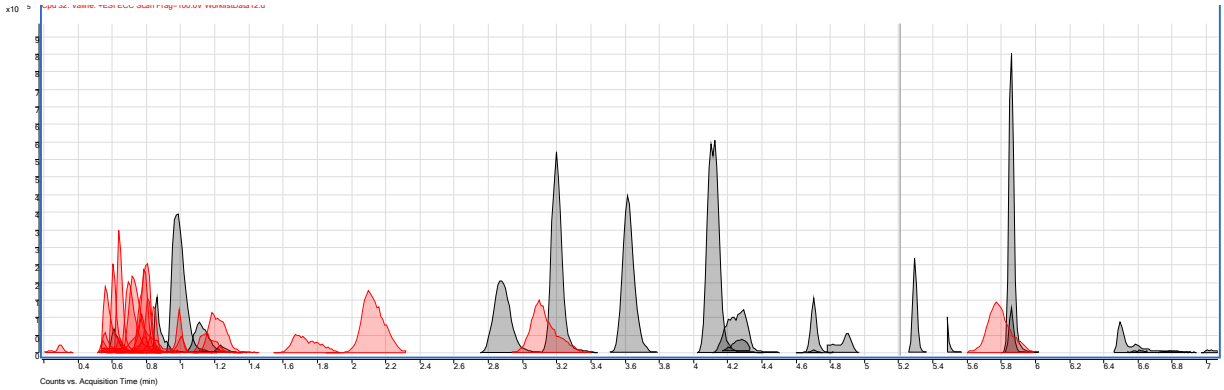
Red color – F5 column, black color – HILIC column

Fig. 34: Overlap chromatogram of standard mixture sample MIX1, F5 column and MIX2, HILIC column

Red color – F5 column, black color – HILIC column

Individual and overlap chromatograms show the separation efficiency of both columns. Schemaof selected peaks show that compounds are better separated by HILIC column.

Fig. 35: Schema of peaks same identified compounds in F5 and HILIC column



Red color – F5 column, black color – HILIC column

PART 4 - Conclusion

9 Conclusion

The samples were identified using the method of peak comparing based on creating standard sample and a database of selected substances. Identification was based on the mass (m/z) value and retention time. Quality of peaks was compared with examined sample and standard sample. The method calibration wasn't without problems.

One of the problems was to limit amount of results in the output to get them suitable and also how to assure the program ranked and named identified substances correctly. A database of own requested structures was found to verify identification. Another possibility for reducing numerous results could be set of optional searching parameters e.g. retention time or m/z.

The next issue was automatically searching identified wrong or fabulous peak or couldn't find requested peak at all. Operating software searching parameters performs great role in identification. Manual search was applied for additional parameter adjustment to current specific peaks found wrong.

pH of the prepared sample didn't have effect on its own analysis or results, chromatograms were comparable.

Reason, why automatic searching wasn't always accurate (found fabulous or no peak), was setting the peak spacing tolerance in automatic searching for 5 ppm. The difference in peak spacing tolerance m/z was greater than tolerance 5 ppm. Automatic searching didn't find peak. Manual searching with enhanced tolerance and difference set to higher value was followed. In value of 10 ppm peak was sliced and in value of 20 ppm was correctly displayed e.g. phenylalanine in standard mixture sample MIX2, HILIC column.
Plasma and standard mixture using F5 column were measured by different conditions – by different time and the newly prepared mobile phases. This was probably reason peaks in plasma sample S2 and standard mixture sample MIX1 had different retention time.

Characterization of substances use mass value only in this method. Properly it can be updated by adding another parameter search e.g. retention time. For better evaluation tandem mass spectrometry (MS/MS) can be used. Information about substances in database can be expanded or updated. The separation took accurate results in HILIC column, compounds were better separated.

10 Souhrn práce

Cílem projektu bylo vyvinout způsob identifikování a porovnání vzorků na základě databáze obsahující několik tisíc známých látek. Navrženou metodou je možné provádět metabolomické analýzy vzorků, čili stanovení metabolitů a toxických látek v biologických vzorcích jako je plasma, moč atd. Metabolomika má široké uplatnění v klinické praxi, diagnostice onemocnění či pro určení biomarkerů narušených metabolických drah vlivem nemoci nebo léčby.

Projekt zahrnoval analýzu lidské plasmy pomocí ultra-účinné kapalinové chromatografie kombinované s hmotnostní spektrometrií a analýzu pomocí operačního programu Qualitative Analysis MassHunter Acquisition Data. V prvním kroku bylo nutné vyhledat pomocí softwaru přítomné látky v plasmě za pomocí modulu automatického vyhledávání a identifikace látek. Za zdroj informací identifikujících látky, jako molekulová hmotnost, m/z, retenční čas, byla použita databáze METLIN, obsahující několik tisíc látek. Bez omezení parametrů pro vyhledávání bylo vygenerováno nadměrné množství dat, které bylo nemožné přehledně zpracovat. Pro vyvinutí postupu standardizace metody byly vybrány základní látky běžně se vyskytující v plasmě (mezi nimi aminokyseliny) a byla vytvořena menší databáze obsahující pouze 97 látek. Parametry automatického vyhledávání a identifikace byly omezeny, jak je tomu uvedeno v kapitole 7.3. Hlavní identifikační parametr byla molekulová hmotnost zahrnutá v databázi. Výsledky vyhledávání jsou uvedeny v tabulkách 5,6,8 v kapitole 7.3.

Druhý krok ověřoval správnost automatické identifikace látek. Za tímto účelem byl vytvořen standardní vzorek látek zahrnutých do vytvořené databáze a uvedených v tabulkách 7 a 9 kapitole 7.3. Postup byl založen na porovnávání retenčního času a kvality píku. V případě, že modul automatického vyhledávání a identifikace látek označil 2 a více píků za stejnou látku, musely být zhodnoceny z hlediska hodnot m/z (látky byly přítomné ve formě iontů $[M+H]^+$ nebo $[M+Na]^+$). V opačném případě by se jednalo o rozdílné látky s jinou hodnotou retenčního času.

Vyhodnocení identifikace nebylo bez problémů. Bylo možné, že pík zkoumané látky nebyl nalezen vůbec, nebo byl nalezen chybně, i přes vysokou koncentraci látky ve vzorku, viz kapitoly 8.4.2 a 8.4.3. Důvodem byla pravděpodobně nastavená hodnota parametru „peak spacing tolerance“ na povolenou odchylku 5 ppm. Konkrétní píky pak bylo nutno vyhledat pomocí modulu manuálního vyhledávání za ruční nastavení požadované hodnoty m/z a toleranci 10 nebo 20 ppm.

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